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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Improved molecular diagnostic methods that can classify tumors and predict their response to therapy have enormous potential to improve the effectiveness breast cancer treatments. The overall goal of this project is to develop a novel molecular diagnostic method, termed SH2 profiling, that can classify cell samples based on their global protein tyrosine phosphorylation state. The first aim is to use an existing SH2 profiling method, based on far-Western blotting, to analyze fresh surgical breast cancer samples. The second aim is to develop a more high-throughput quantitative reversed-phase SH2 profiling format, and test its usefulness in classifying breast cancer samples. The third aim is to develop histochemical SH2 profiling methods that can be used to analyze archived, formalin-fixed tissue sections, and perform pilot retrospective studies to determine whether SH2 binding patterns have potential prognostic value. In the past year we have made great progress in developing the reversed-phase array and histochemical SH2 profiling formats, and results suggest that these quantitative methods will be useful in classifying breast cancer samples. In the coming year, once HSRRB approval for use of human samples is secured, we will begin to apply these new methods to the analysis of actual breast cancer specimens.

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INTRODUCTION

The focus is this study is to test whether a novel molecular diagnostic approach, SH2 profiling, can serve as a useful prognostic tool to classify breast cancer. SH2 domains are small protein modules that bind specifically to tyrosine phosphorylated peptides. These domains play an important role in normal signal transduction, mediating the formation of multiprotein complexes in response to changes in tyrosine phosphorylation [1, 2]. There are ~116 SH2 domains in the human genome, and different SH2 domains recognize different tyrosine phosphorylated proteins. SH2 profiling is a method in which a battery SH2 domain probes is used to provide a snapshot of the global state of tyrosine phosphorylation in a cell sample [3, 4]. Different breast cancers are likely to have different patterns of tyrosine phosphorylation, reflecting differences in the signal transduction pathways active in the tumor, and thus SH2 profiling has the potential to provide a biologically relevant means to classify these tumors. In this project we proposed to test different formats of SH2 profiling (far-western blotting, reverse-phase arrays, histochemical analysis of fixed tumor sections) using breast cancer samples to test the feasibility of these approaches and their potential utility as prognostic tools.

BODY

Task1: Perform SH2 profiling on fresh surgical samples of invasive carcinoma of the breast. Correlate SH2 profile data with standard pathologic criteria and other known molecular markers.

Because of unforeseen difficulties in reconciling the human subjects research protocol previously approved by the UCHC Institutional Review Board with the requirements of the US Army Human Subjects Research Review Board, this project has not yet received final approval for studies involving human surgical samples. The final required paperwork has now been submitted, and we anticipate receiving approval some time in August 2004. During the past year we have also initiated a collaboration with surgeons at Hartford Hospital to provide surgical breast cancer samples for this study under the approved UCHC protocol. Because we were expressly forbidden to perform experiments outlined in this task, we have no progress to report at this time.

Task 2: Develop reverse-phase protein microarray assay to quantitatively analyze SH2 binding profiles of patient tumor samples

a) Test approach using macroarrays of lysates of cell lines (months 1-6)
b) Test miniaturization of assay to microarray format—detection by ECL (months 7-12)
c) Apply to tumor samples of Aim 1 (years 2 and 3)
d) Test fluorescent SH2 labeling and detection (year 2)

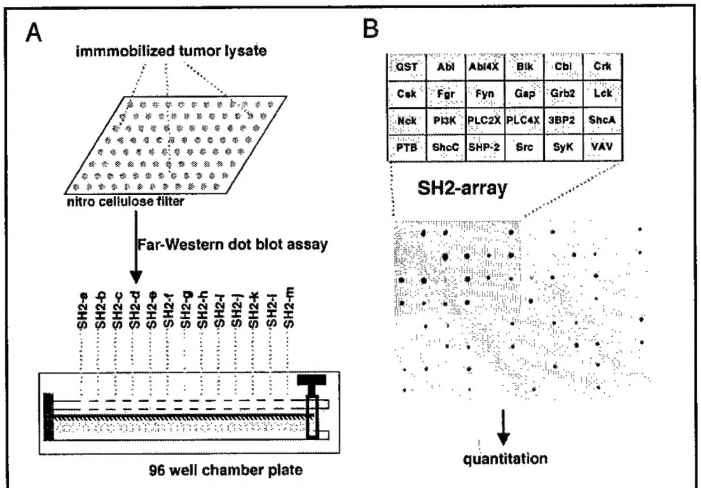

Fig. 1. Reverse-phase SH2 array. (A) Identical aliquots of protein sample are spotted on membrane using 96-well chamber plate. After drying and blocking the membrane, each well is incubated with a different labeled SH2 domain. (B) Representative reverse-phase array data. Each quadrant of a membrane was probed with 24 GSH-HRP-labeled SH2 domains (indicated diagrammatically at top) and binding detected by ECL.

Tasks 2a and b: We have devoted considerable effort to developing a robust, reproducible, and quantitative reverse-phase "dot-blot" assay that can be used to generate SH2 profiling data from complex protein mixtures (cell or tissue lysates) as well as purified phosphoproteins. The current assay format uses a 96-well blotting apparatus. Identical aliquots of protein samples are spotted on nitrocellulose or PVDF membranes in each well of the apparatus, and following blocking each well is loaded with a different labeled SH2 domain probe (Fig. 1). After washing, bound SH2 domains are detected by ECL, exposed to X-ray film, and quantitated using NIH-Image software. Alternately signals can be quantitated using a phosphorimager. Multiple protein samples can be spotted in each well (at least 6 different samples/well), allowing relative binding of each sample to different SH2 domains to be assessed. The throughput of this system is relatively high, as six different samples can be quantitatively assayed for binding to up to 96 different SH2 or antibody probes in a matter of several hours. Fig. 2 shows representative data for mouse fibroblasts

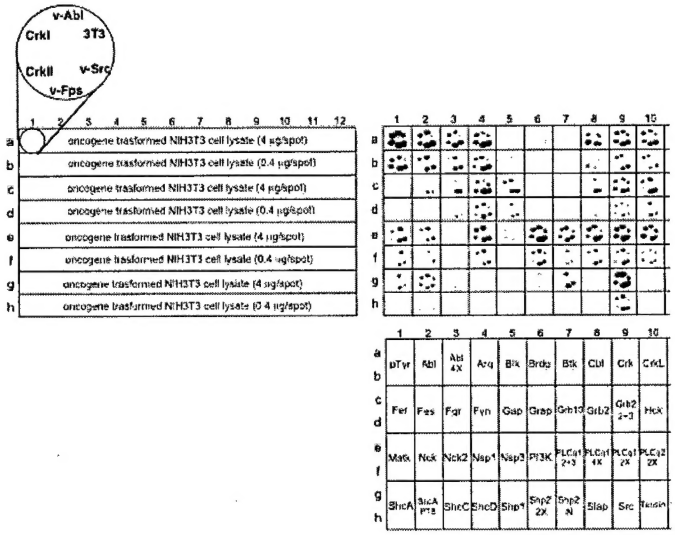

Fig. 2. Representative reverse-phase SH2 binding data. Lysates of NIH-3T3 cells transformed with various oncogenes were replica spotted on membranes as indicated in upper left, at two different sample concentrations. Each well was then incubated with different labeled SH2 domain probes as indicated in lower right, and binding detected by ECL. Different cell samples show different patterns of SH2 domain binding (upper right).

transformed with different oncogenes, probed with 46 different SH2 domain probes plus controls. The results demonstrate that the different transformed lines have distinct quantitative patterns of SH2 binding. These results are consistent with 1D far-Western analyses of the same lysates (Fig. 3). These results validate the macroarray SH2 platform and suggest that different breast cancer samples may also be differentiated based on their quantitative SH2 profiles.

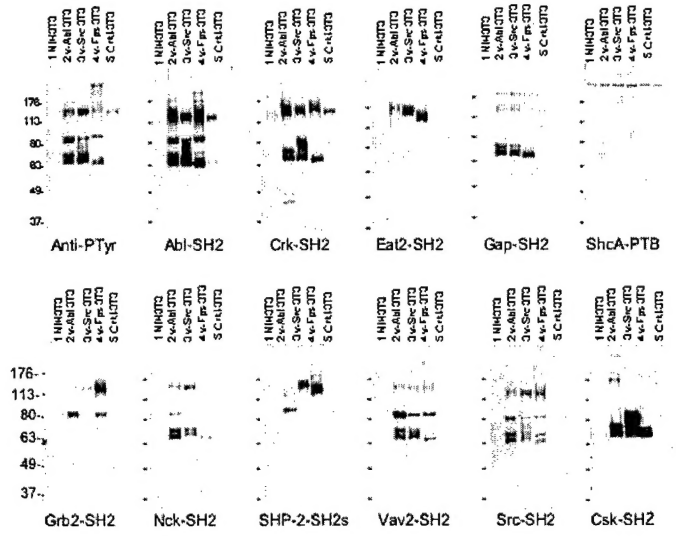

Fig. 3. Far-western blotting of protein samples analyzed in Fig. 2. Protein lysates of NIH-3T3 cells transformed with various oncogenes were separated by SDS-PAGE and transferred to membranes. Identical membranes were probed with labeled SH2 domains as marked and bound probe detected by ECL. Each cell lysate has a different qualitative and quantitative pattern of SH2 binding.

We should note that these macroarray experiments were performed under noncompetitive binding conditions. Competition with unlabeled SH2 domains is expected to increase specificity and thereby increase discrimination among profiles[4]. Competition with ethacrynic acid-blocked SH2 domains is technically challenging and dependent on experimental conditions (unpublished observations). However we anticipate that when enzymatically biotinylated SH2 domains are available (see below), competitive reverse phase array data will provide greater quantitative differences in SH2 binding patterns.

We have also spent considerable effort to increase the number of SH2 domain probes available for use in SH2 profiling studies. There are ~116 total SH2 domains in the human genome [1], and during this funding period we have constructed GST fusions for ~40 previously unavailable SH2 domains, and upon purification and testing found that ~15 of these are now suitable for profiling assays (i.e. give detectable signal and low nonspecific background in far-Western binding assays of whole cell lysates).

We have not yet tested the miniaturization of this assay to a microarray (nitrocellulose-coated slide) format, because we do not yet have access to the large number of clinical samples that would make such a format potentially useful. Furthermore, given the success of the macroassay described above and the relatively small amounts of sample it requires, as well as the ease with which multiple probes can be simultaneously quantitated in the same experiment, we felt that the effort to establish

the microarray format was not currently justified. However the macroarray experiments are giving us valuable information regarding sample preparation, blocking, binding and washing conditions, and data analysis that could easily be applied to the microarray format in the future.

Task 2c will be performed once clinical samples become available. For Task 2d, we have not explicitly produced fluorescent probes, but we have established a system in which GST-SH2 fusion proteins are expressed with a C-terminal "avitag", which directs enzymatic biotinylation by the BirA product in *E. coli* [5]. Each SH2 probe is thus tagged with a single biotin moiety, eliminating the disadvantages of heterogeneous multiple biotinylation seen when chemical biotinylation is performed. These singly-biotinylated probes could easily be stoichiometrically labeled using fluorophore-conjugated streptavidin for detection of binding in microarray formats using standard fluorescent array readers. In addition, the biotinylated probes will greatly simplify competitive binding assays, as unbiotinylated SH2 domains can be used instead of ethacrynic acid-blocked competitors, which are necessary when GSH-HRP conjugates are used as probes.

Task 3: Establish conditions for using SH2 domains to probe sections of paraffin-embedded tumor samples. Perform retrospective study using archived tumors to test if positive staining with specific SH2 probes correlates with recurrence or survival.

a) Establish histochemical staining method for detection and quantitation of binding of SH2 domains to sections of tumors (year 1)
b) Analyze ~100 archived samples per year with at least 5 SH2 domain probes (ongoing; aim is to analyze at least 200 by end of year 2)
c) Establish database with pathological criteria, patient outcomes; SH2 binding data when available (months 1-6)
d) Correlate SH2 binding with standard pathological criteria (months 6-24)
e) Correlate SH2 binding with patient outcomes (recurrence, mortality) (years 2 and 3)

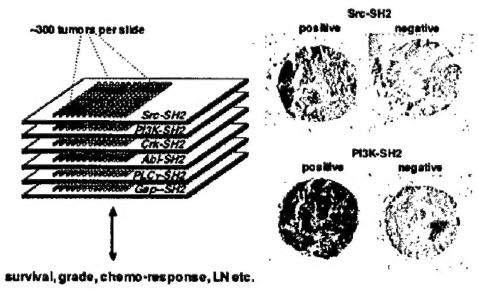

Fig. 4. Tissue Microarray format. Blocks containing multiple arrayed tumor samples are sectioned; each section is probed with a different SH2 domain and binding detected by DAB staining (brown). On right, representative positive and negative samples shown for two SH2 probes.

Work on Task 3 was initially delayed by lack of approval by the HSRRB to perform research on human anatomic samples. Approval was ultimately granted for these experiments in late November, 2003. Since that time we have made some effort to optimize conditions for increasing signal strength and decreasing nonspecific background binding when SH2 domains are incubated with fixed tissue sections. We also initiated collaborations with Dr. Harriet Kluger and David Rimm of Yale University medical school, who are experts in tissue

microarray analysis (TMA) and who have assembled several large tissue arrays of matched breast carcinoma samples are associated with long-term clinical outcomes data. While the TMA studies are not explicitly covered by the current DOD grant, it is obvious that results of such experiments will support the retrospective studies on samples from the UCHC pathology archives, and that methods optimized in either platform can be used interchangeably in each (Fig. 4).

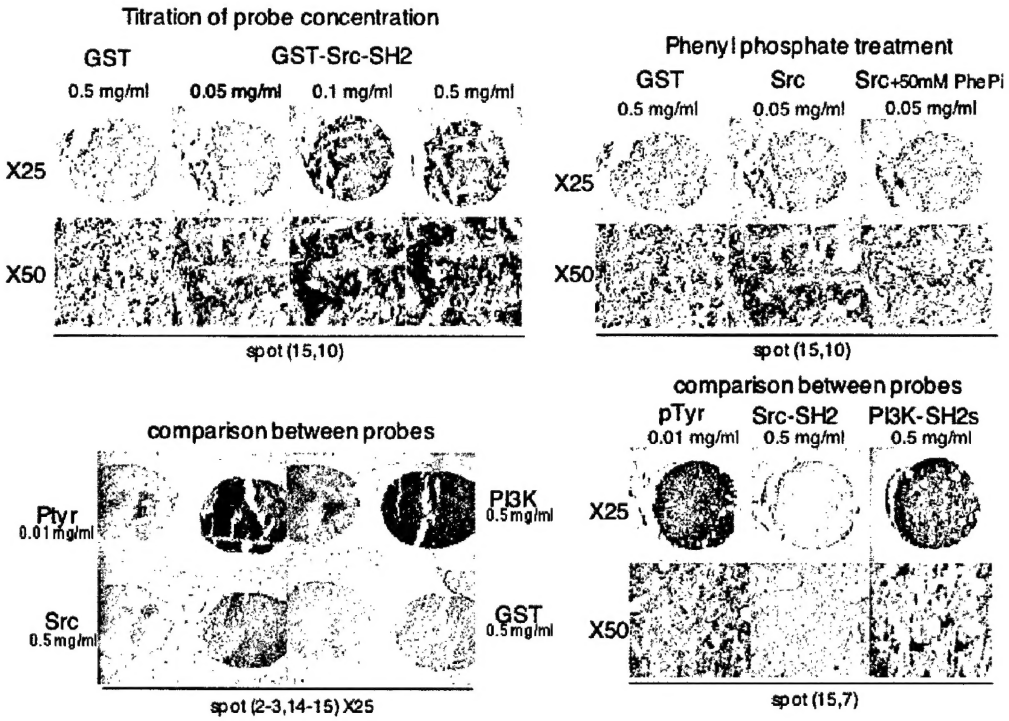

Fig. 5. Validation of histochemical staining with SH2 domain probes. Tumor samples in tissue microarray format were incubated with GST-SH2 domain probes and detected with GSH-HRP and DAB (brown indicates positive staining). Upper left: signal increases with increasing concentration of SH2 probe, while GST control remains negative. Upper right: co-incubating SH2 probe with phenyl phosphate, a pTyr analog, diminishes binding, indicating binding is pTyr-dependent. Lower panels: representative examples of the sections of same tumor stained with different SH2 domains or anti-pTyr (positive control for presence of pTyr) or GST (negative control). Some tumors bind strongly to a subset of SH2 domains (for example, on lower right tumor 15-7 binds strongly to the PI3K SH2s but not to the Src SH2 probe).

In these pilot studies, we have found that relatively high levels of SH2 probe are necessary (Fig. 5), and that it is important that GSH-HRP label be added after binding of GSH-SH2 probes, not prior as in the case of far-western blotting of membranes. This is likely because the size of the complex of GSH-SH2 and HRP-conjugated GSH is too large to easily enter the fixed tissue. We also demonstrated that co-incubation of tissue sections with the phosphotyrosine mimetic phenyl phosphate almost completely eliminates the signal from SH2 binding, indicating that the signal is specific to tyrosine-phosphorylated proteins as expected (Fig. 5). Preliminary studies in the TMA format also showed that individual tumor samples can be strongly positive with one SH2 domain probe and anti-pTyr, but not bind strongly to other SH2 domain probes (Figs. 4 and 5),

demonstrating that different tumors have different patterns of SH2 binding, and further suggesting that this approach will be useful in classification of tumor samples.

Dr. Poornima Hegde, of the Department of Anatomic Pathology at UCHC, is currently assembling matched panels of invasive carcinoma of the breast, varying in Her2/Neu amplification and in their ER/PR status. These samples will be used in initial experiments to determine if binding of different SH2 domains correlates with these standard prognostic criteria. Once these pilot studies are accomplished in the next several months, we can proceed to larger analyses of ~100 tumor samples per year as outlined in the SOW. Because we are still in the stage of protocol optimization and troubleshooting, we have not yet established a database of outcomes data and SH2 profile data for tissue blocks in the UCHC archive. However we have established a collaboration with Ms. Laura Glynn of the UCHC General Clinical Research Center who is the study coordinator for other ongoing SH2 profiling studies, and who will maintain a similar database (with patient identification coded) for breast cancer samples.

KEY RESEARCH ACCOMPLISHMENTS

•Development of reproducible, robust reverse-phase macroarray platform for SH2 profiling and its validation using cell lysates
•Generation and purification of ~40 new SH2 domain probes, ~15 of which are suitable for use in SH2 profiling assays
•Establishment of standard conditions for specific binding of SH2 domain probes to fixed tissue sections
•Demonstration in Tissue Microarray experiments that different tumor specimens can have different profiles of SH2 domain binding, validating this approach as a possible method for classifying breast cancers

REPORTABLE OUTCOMES

Research Abstract: Kazuya Machida, Christopher M. Thompson, and Bruce J. Mayer. Proteomic profiling of SH2 domain binding. Abstract for platform presentation at the 20th Annual Meeting on Oncogenes, Frederick MD, June 16-20, 2004.

Review Article: Machida K and Mayer BJ. The SH2 domain: versatile signaling module and pharmaceutical target. Biochim Biophys Acta Proteins and Proteomics, manuscript submitted.

CONCLUSIONS

The work over the past year has strongly suggested that SH2 profiling will prove to be a useful method for classifying breast cancers. Because work with human anatomic samples was not permitted during the early stages of the project, our efforts focused on developing assays and reagents so we will be prepared to efficiently perform profiling experiments once clinical samples are available. The reverse-phase assay will be critical

for eventual clinical use of SH2 profiling, since it will probably not be economically and technically feasible to perform 1-D far-western assays as a routine diagnostic procedure. Critical elements for a reverse-phase array are low background, high reproducibility, and ability to use small amounts of sample. All of these are satisfied by the current macroarray format, so we do not anticipate adapting this assay to a true microarray format unless we encounter problems with sample amounts or throughput in future studies with surgical samples. We also have been working to establish conditions for labeling of formalin-fixed pathological samples using SH2 domains. Again acceptable conditions have been found, and preliminary tissue microarray experiments suggest that such assays can serve as a basis for classification. This is very promising, because retrospective assays of archived tumor samples, associated with clinical outcomes data, will rapidly indicate whether classification that is based on SH2 binding has any prognostic value. Finally, ongoing work to generate more SH2 domain probes, and to adapt them for stoichiometric labeling with biotin using the BirA system, will contribute greatly to the overall usefulness of this approach in classification of tumors. With these tools (methods and reagents) in place, we are now well positioned to perform the critical experiments on human samples that will definitively establish the prognostic value of SH2 profiling of breast cancers.

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APPENDICES

1. Abstract for 20th annual Meeting on Oncogenes, Frederick MD (June 16-10, 2004)

2. Manuscript of solicited review article by K. Machida and B.J. Mayer submitted to *Biochimica et Biophysica Acta Proteins and Proteomics*

Abstract, presented at 20th Annual Meeting on Oncogenes, Frederick MD June 16-20, 2004

Proteomic profiling of SH2 domain binding

Kazuya Machida, Christopher M. Thompson, and Bruce J. Mayer
Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, CT

Protein tyrosine phosphorylation plays a key role in a variety of cell signaling pathways, regulating activities such as proliferation, differentiation, morphological change and transformation. Tyrosine phosphorylation of proteins can create binding sites for downstream signaling molecules via phosphotyrosine-recognizing domains such as Src homology 2 (SH2) domains and phosphotyrosine binding (PTB) domains. We employ a battery of SH2 domains as probes to profile the global cellular tyrosine phosphorylation state of the cell (termed SH2 profiling). Using methodologies based on the far-Western binding assay in dot blot, 1D gel and 2D gel formats, we have applied SH2 profiling to a variety of cell/tissue systems. Current efforts are underway to profile SH2 binding patterns in 1. Cells transformed by oncogenic protein tyrosine kinases (PTKs), 2. Tyrosine phosphorylated site(s) of specific signal transduction proteins, 3. Changes associated with stimulation/differentiation of cells, including myelin oligodendrocytes (OLs), and 4. Classification of patient samples of chronic myelogenous leukemia (CML) and breast cancer. The results suggest that a reversed-phase SH2 array format can rapidly discriminate among different PTK-transformed cells and SH2 binding sites. Although the sensitivity of detection in 2D-far-Western assays is not as great as in 1D format, we found that SH2 domains could successfully detect changes in tyrosine phosphorylation of specific protein spots in stimulated OL lysates. We are now well on our way to assembling a complete set of all human SH2 domains and PTB domains, which can be used to generate comprehensive phosphoproteomic maps of PTK signaling pathways in different cells.

Solicited review manuscript submitted to *Biochimica et Biophysica Acta Proteins and Proteomics*

The SH2 domain: versatile signaling module and pharmaceutical target

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Running title: SH2 domains and inhibitors

Keywords: protein tyrosine kinase; signal transduction; SH2 inhibitor; phosphotyrosine mimetic

Summary

The Src homology 2 (SH2) domain is the most prevalent protein binding module that recognizes phosphotyrosine. This ~100-amino-acid domain is highly conserved structurally despite being found in a wide variety proteins. Depending on the nature of neighboring protein module(s), such as catalytic domains and other protein binding domains, SH2-containing proteins play many different roles in cellular Protein Tyrosine Kinase (PTK) signaling pathways. Accumulating evidence indicates SH2 domains are highly versatile and exhibit considerable flexibility in how they bind to their ligands. To illustrate this functional versatility, we present three specific examples: the SAP, Cbl and SOCS families of SH2-containing proteins, which play key roles in immune responses, termination of PTK signaling, and cytokine responses. In addition, we highlight current progress in the development of SH2 domain inhibitors designed to antagonize or modulate PTK signaling in human disease. Inhibitors of the Grb2 and Src SH2 domains have been extensively studied, with the aim of targeting the Ras pathway and osteoclastic bone resorption respectively. Despite formidable difficulties in drug design due to the lability and poor cell permeability of negatively-charged phosphorylated SH2 ligands, a variety of structure-based strategies have been used to reduce the size, charge and peptide character of such ligands, leading to the development of high-affinity lead compounds with potent cellular activities. These studies have also led to new insights into molecular recognition by the SH2 domain.

1. Introduction

The Src homology 2 (SH2) domain is the prototype for protein-protein interaction modules that mediate the formation of multiprotein complexes during signaling [1, 2]. Unlike other binding modules such as the SH3 or PDZ domains, SH2 domains specifically function in protein tyrosine kinase (PTK) pathways, due to the dependence of their binding on tyrosine phosphorylation. They are evolutionarily conserved in many eukaryotes [3], and ~116 different examples (representing ~106 distinct proteins) are found in the human genome [4]. In addition to the phosphorylated tyrosine (pTyr) residue, each SH2 domain recognizes several additional flanking residues, usually three to five amino acids C-terminal to pTyr, thereby acquiring selectivity for specific phosphorylated sites. For example, the autophosphorylated cytoplasmic domain of the PDGFβ receptor provides distinct binding sites for several specific SH2-containing proteins, including Y579 (*po*YIYV, where *po*Y denotes phosphotyrosine [5]) for Src and its relatives; Y740 (*po*YMDM) for phosphatidylinositol 3-kinase (PI3K); and Y771 (*po*YMAP) for Ras-GAP. Combinatorial phosphopeptide libraries have been used to probe the binding preferences of SH2 domains, leading to the realization that SH2 domains can be divided into classes based on their sequence and binding preferences [6, 7].

In the cell, the specific association of SH2 domains and tyrosine phosphopeptides is used to mediate the reversible relocalization of proteins, which is important for efficient propagation of PTK signals [8]. SH2 domains are usually found in proteins together with other modules, either catalytic domains (for example, protein or lipid kinase and phosphatase domains), or other protein binding modules (for example SH3, PH, or PTB domains). Thus, depending on the nature of those other modules, proteins containing SH2 domains can participate in a wide variety of intracellular functions (reviewed in [2, 8-11]). For instance, Grb2 (which contains two SH3

domains in addition to one SH2 domain) functions to recruit the downstream regulators SOS and Gab1 following receptor tyrosine kinase (RTK) activation. In this example, the Grb2 SH2 domain binds to sites on the autophosphorylated RTK, leading to Ras activation via SOS, which is bound to the N-terminal SH3 domain, while the PI3 kinase-Akt pathway is activated via Gab1, which is bound to the C-terminal SH3 domain [10]. When the other domains include an inhibitory module, as in the case of Cbl or SOCS, SH2-containing proteins can also act as negative regulators of signaling (discussed below, Section 2).

In addition to relocalizing proteins to specific sites (binding in trans), interplay between an SH2 and other domains in the same molecule (in cis) can also be important. SH2 domains of the Src and Abl families of nonreceptor tyrosine kinases engage in intramolecular interactions that maintain the kinase in an inactive configuration [12]. Association of the SH2 domains with extrinsic ligands relieves the autoinhibitory interaction, triggering catalytic activation. Similarly, activation by SH2 domain ligands has been reported for proteins containing tandem SH2 domains such as SHP-1, SHP-2, PI3K, Syk, ZAP-70 and PLCγ, probably because the global protein conformation is altered upon ligand binding [13-19]. In the case of nonreceptor tyrosine kinases, the SH2 and kinase domain also cooperate to enhance signals by facilitating processive phosphorylation of multiple tyrosines on substrate proteins [20-22].

SH2 domain function relies on two principles, tyrosine phosphorylation dependency and ligand specificity, whose structural basis is well understood. An SH2 domain consists of ~100 amino acids with two α-helixes and seven β-strands, ordered βαββββββαβ, with a central anti-parallel β-sheet flanked by α-helices [23] (Fig. 1). Tyrosine-phosphorylated ligand peptides are recognized by two key regions, one the pTyr binding site (referred as the pTyr pocket), and a so-called "specificity determining region" which interacts with ligand residues C-terminal to pTyr.

Based on X-ray crystal structure studies of the Src and Lck SH2 domains bound to high affinity ligands, the typical SH2 binding mode has been likened to a two-pronged plug engaging a two-holed socket, where the peptide backbone is extended and the sidechains of pTyr and the Y+3 residue each project deeply into complementary pockets on the SH2 surface [24, 25]. Different binding modes have also been observed; for instance the Grb2 SH2 prefers ligands with a β-turn conformation of the peptide backbone, in which the Y+2 Asn residue plays a critical role [6, 26] (various binding modes are reviewed in [27]).

Although the two-pronged model is a useful description of SH2 binding, the binding energies conferred by the pTyr pocket and the specificity-determining region are not equivalent. It is appropriate that the pTyr-dependent interaction should dominate, to ensure that the domain acts as a phosphorylation-dependent molecular switch. As a consequence, the selectivity of a single SH2 domain for its cognate peptide motif is not absolute. The in vitro dissociation constant for binding of an SH2 domain to its preferred pTyr peptide ranges from 0.2 to 1 μM, over four orders of magnitude greater than the affinity for the unphosphorylated counterpart, but only moderately higher (~30-150 fold) than for non-specific pTyr peptides [6, 28-31]. It is important to remember that physiologically relevant ligands may not necessarily conform to the "optimal" high-affinity ligands obtained from in vitro screens [32]. Moderate specificity interactions can be favored by high local concentration, or by the interaction of other domains of the two partners. In other cases, recognition of overlapping sets of target peptides by SH2 domains may be important to allow signals to amplify or diverge, creating multiplexed signaling networks.

The peptide binding specificity of an SH2 domain can be switched by point mutation of a key residue in the specificity determining region [33-35]. For instance, a Thr to Trp mutation at

residue EF1 of the Src SH2 converted its peptide preference to that of Grb2 [34] (here and throughout, the established nomenclature for SH2 domain residues is used, where strands and helices are denoted by letter; see Fig. 1) [25]). More recently it has been suggested that many SH2 domains, including the unmutated Src SH2, are able to bind quite well to typical Grb2 ligands [36]; however in the case of the Grb2 SH2, the presence of the bulky Trp side chain prevents binding of extended peptides and thus selects exclusively for *po*YxNx motifs that adopt a β-turn confirmation [35]. Thus SH2 binding specificity and binding mode appear to be more flexible than initially thought. Indeed, although SH2 binding is entirely dependent on tyrosine phosphorylation in most cases, ligands lacking pTyr have also been reported, including serine- or threonine phosphorylated sites [37-40], phosphatidylinositol(3,4,5) trisphosphate [19, 41], and unphosphorylated ligands [42-45]. This suggests that some SH2 domains might have evolved in an ad hoc fashion to recognize, with moderate affinity, various negatively charged ligands.

Relative to single SH2 domains, the bidentate binding of tandem SH2 domains to bisphosphorylated peptide ligands, as in the binding of the p85 subunit of PI3K to activated PDGFβ receptor doubly phosphorylated on Y740 and Y751, confers substantially higher affinity and specificity. Such an arrangement may indicate a requirement for unambiguous molecular recognition to elicit the appropriate biological response [46]. The increased specificity of multivalent binding can also be seen when SH2 domains act in concert with other binding module(s), for example an SH3 domain, to trap a ligand that bears binding sites for both domains [47-49].

Taken together, it is apparent that SH2 domains are diverse and versatile in nature, capable of either promiscuous or strict specificity depending on the modular domain arrangement and cellular context. It is worthwhile discussing in greater detail a few specific cases that

illustrate these diverse functions. In the next section, we describe recent work on three interesting families of SH2 proteins: SAP, Cbl and SOCS. In the following section we highlight efforts to develop pharmaceutical SH2 inhibitors targeting PTK signaling in human disease.

2. SH2 domains in signal transduction

2.1 SH2 domain as blocker or adaptor?

X-linked lymphoproliferative disease (XLP) is a recessive hereditary disorder characterized by inadequate immune response to Epstein-Barr virus (EBV) infection, resulting in organ damage and death [50-52]. Abnormal T cell, B cell, and NK cell responses to virus infection have been reported in XLP, yet the mechanism of molecular pathogenesis has been elusive [52]. In 1998, three different groups independently identified the same gene (variously named *SAP/SH2D1A/DSHP*) which is mutated in XLP [42, 53, 54]. Surprisingly, the gene product SAP consists almost entirely of a single SH2 domain, flanked by a mere five N-terminal and 26 C-terminal residues lacking any recognizable functional motifs (Fig. 2A). In T cells, SAP was shown to bind to a tyrosine residue (Y218) in the cytoplasmic region of the receptor SLAM/CD150, suggesting a role in receptor signaling [42].

Unlike typical SH2 domains, the SH2 domain of SAP can bind the unphosphorylated Y281 site of SLAM with high affinity (K_d of 650 nM); the affinity increases only 4-5 fold upon phosphorylation [42, 55, 56]. Interestingly, the X-ray crystal structure of the ligand-bound form of SAP revealed that the binding could best be described as a "three-pronged plug and socket," with three distinct regions mediating specific interactions. Phosphopeptide library screening indicated the consensus motif for SAP binding is TI*po*Yxx(V/I) (where x represents any amino acid); the Y-2, pTyr, and Y+3 positions are most important, consistent with the three-pronged

model. Moreover, results suggested that any one of the three was dispensable for high-affinity binding, as long as the other two determinants matched the consensus [55-58]. Consistent with this, the tyrosine residue of the SLAM Y281 peptide can be replaced by any amino acid [58]; however, it should be noted that physiological ligands without tyrosine at the corresponding residue have not yet been identified.

How can this unique binding mode be explained structurally? The overall structure of SAP is similar to other SH2 domains such as Src [24]; the pTyr-pocket is on one side of the central antiparallel β sheet while a hydrophobic groove on the other side interacts with the C-terminal part of the ligand peptide. However three unique features were seen in the structure of SAP bound to the SLAM Y281 peptide [56, 58]. First, the residues N-terminal to tyrosine make specific contacts with the SH2: a parallel β sheet interaction with strand βD of the domain, assisted by hydrogen bonds from Thr at Y-2 of the ligand with GluαA6 and with a buried water molecule. Second, the unphosphorylated Tyr residue associates with ArgβB5 (the "FLVRES" arginine, which is conserved in the pTyr pocket of almost all SH2 domains) via ordered water molecules instead of the usual phosphate oxygens. Interestingly, EAT-2, a molecule closely related to SAP, also binds the Y281 SLAM peptide in a three-pronged fashion. However in this case binding is phosphorylation-dependent, possibly because the more hydrophobic environment of the EAT-2 pTyr pocket relative to SAP (eliminating a buried water molecule) reduces interactions with unphosphorylated tyrosine [59]. Third, the C-terminal hydrophobic pocket is rather flexible and can adopt both open and closed configurations depending on the C-terminal residues of the ligand. This pocket is closed off by the EF loop when the groove is not occupied, creating a ridge to accommodate ligand peptides lacking a C-terminal "prong."

How then does this simple SH2 protein play an important role immunoreceptor signaling relevant to XLP? Two models have emerged: SAP acts either as a competitor or as an adaptor. A single SH2 domain may serve as a natural competitor for other SH2-containing proteins by preventing their binding to sites such as Y281, Y307, and Y327 of SLAM [60]. SHP-2, a protein tyrosine phosphatase (PTP) with tandem SH2 domains, has been repeatedly coimmunoprecipitated with SLAM [42, 55, 61-63]. This interaction is disrupted by co-transfection of SAP, suggesting that SAP competes with SHP-2 for binding to phosphorylation sites on SLAM. Indeed, Y281 and Y327 match the peptide consensus for both SAP and SHP-2 (Fig. 1C) [56, 58, 64].

Second, SAP may have an adaptor function. Forced expression of SAP induces tyrosine phosphorylation of SLAM and coprecipitation of SLAM with SH2- or PTB-domain-containing proteins such as SHIP, Dok2, Dok1, Shc, and Ras-GAP. Thus it appears that SAP may not compete with these other proteins for binding to SLAM, but instead facilitates their association to the receptor [63, 65]. Importantly, this effect requires coexpression of FynT, a T cell-specific isoform of the Src family tyrosine kinase Fyn; on the other hand it is apparently phosphatase-independent, because PTP inhibitors do not alter the tyrosine phosphorylation level. Moreover, FynT has been shown to bind to SLAM in a SAP-dependent manner. Taken together, these findings strongly suggest that SAP functions as an adaptor, bridging FynT and SLAM. The mechanism was obscure, however, given the single-domain structure of SAP [66].

A potential solution to this conundrum was reported simultaneously by three groups in 2003: the SH2 domain of SAP could be shown by biochemical and structural approaches to bind directly to SH3 domain of FynT [64, 67, 68]. The binding interface overlaps with the canonical ligand binding region of the FynT SH3 domain but not that of the SAP SH2 domain, and the

association was abolished by mutations in the SH3 domain that inactivate its binding to canonical proline-rich peptides [67]. The FynT SH3 domain does not bind the SAP SH2 via a polyproline motif, but instead through a small region of the βF strand and αB helix; mutation of Arg in the FB loop abolished binding to FynT without affecting binding to SLAM [67, 68]. (Parenthetically, this contrasts with the other well-characterized example of trans SH2-SH3 interaction, in which a canonical SH3 binding mode was observed between the Abl SH3 domain and a polyproline motif in the long DE loop of the Crk SH2 domain [69]). Importantly, binding of SAP to the FynT SH3 domain appears to lead to kinase activation of FynT [70], presumably by abrogating SH3-mediated intramolecular autoinhibition as previously observed for other Src family tyrosine kinases.

It should be noted that the two models for SAP function described above are not mutually exclusive [71]. Although SAP is mutated in XLP patients, there is no obvious correlation between XLP disease phenotypes and SAP genotypes or the binding spectra of SAP SH2 domains to immunoreceptors [55, 72]. Interestingly, introduction of several disease-specific missense mutations into SAP attenuates the association of SAP with FynT [64], suggesting a potential role of this interaction in the disease phenotype.

A number of intriguing questions remain. For example, what is the biological relevance of the phosphorylation-independent association of the SAP SH2 with the Y281 site of SLAM? A disease-specific mutant of SAP, T53I, can bind phosphorylated SLAM as well as wild-type (wt) SAP but has much lower affinity for the unphosphorylated form [55], suggesting phosphorylation-independent binding may be important. However T53I SAP also has lower affinity for other partners, such as CD84, 2B4 (CD244), Ly9 (CD229), and FynT, which also may account for pathogenesis [55, 64]. It will also be very interesting to know how binding of

the FynT SH3 domain affects the binding preference of the SAP SH2 domain. Another open question involves competition between SAP and other partners for SH2 binding sites on the SLAM receptor. Why does SAP apparently compete with some partners for binding, such as SHP-2, but not with others such as SHIP? A plausible model is that SAP competes with SHP-2 for binding to Y281, but does not affect association of SHIP with Y307 and Y327 [42, 55, 61-63]. However in vitro binding data indicate that SHP-2 and SHIP bind equally to the Y281, Y307, and Y327 sites of SLAM [64]. Possibly three-dimensional steric factors affect the accessibility of SH2 domains to these sites in the intact proteins. Alternatively, SHP-2 may regulate binding by dephosphorylating its SH2 domain binding site, as shown in the case of SHP-2 binding to IRS-1[73]. The cytoplasmic domain of SLAM-related receptors contain a TxYxx(V/I) motif that has been dubbed the immunoreceptor tyrosine-based switch motif (ITSM), the function of which is proposed to switch depending on its SH2-containing binding partners, such as SAP, SHP-2 or SHIP [63, 74].

Taken together, data suggest that the seemingly simple structure of SAP belies a complex function: by coupling FynT to SLAM, it mediates the tyrosine phosphorylation of sites that can recruit SHIP and other SH2 proteins. SAP (but not EAT-2, which appears to lack a FynT binding motif) has been shown to play a similar role for other immune receptors such as 2B4/CD244, which contain different spectra of SH2 binding sites [71, 75-77]. Since SLAM can modulate production of Th1-type cytokines such as interferon γ and tumor necrosis factor [61, 78], SAP deficiency may lead to abnormal Th1 responses. Indeed, impaired Th2 differentiation was observed in T cells from SAP-/- mice, which manifest up-regulation of interferon γ and down-regulation of IL-4, similar to SLAM-/- T cells [79-81]. The IL-4 defect is more drastic in SAP-/- T cells, however, suggesting SAP may be involved in other SLAM-related receptor pathways.

Mice are not susceptible to EBV infection, so XLP must for now be studied in the less experimentally tractable human system. Although SLAM is generally thought to play a dominant role in mediating T cell cytotoxicity, a recent paper showed very low expression of SLAM and high expression of 2B4 on EBV-specific T cell lines from normal and XLP donors, suggesting a more dominant role for 2B4 in EBV infection [82]. Interestingly, the T cell lines from EBV-exposed and EBV-non-exposed XLP patients showed different interferon γ production levels following CD3 stimulation. Others found correlations between the expression of SLAM, 2B4 and SAP in T cells with clinical and immunological features of patients with EBV infection or tuberculosis [83, 84]. Although XLP pathogenesis appears to be due to a disturbance of the Th1/Th2 cytokine balance, more clinically relevant studies are needed.

2.2 Cbl: inhibitor of Active PTKs

The proto-oncogene Cbl was originally identified as the cellular counterpart of the retroviral oncogene v-Cbl, which induces B-cell lymphoma in mice [85]. The v-Cbl protein was found to have suffered a large C-terminal deletion compared to wt Cbl, which consists of an N-terminal domain (termed Cbl-N), a proline-rich region, and a C-terminus containing multiple potential sites of tyrosine phosphorylation (Fig. 2B). Cbl binds a number of signaling proteins containing SH2 and SH3 domains such as Crk, Grb2, Src and PI3K, and thus was initially thought to be an adaptor/scaffold protein [86-89]. However, three important findings have revealed a very different function for Cbl. First, Sli-1, the Cbl homologue of *C. elegans*, was found to act as a negative regulator of signaling downstream of the Let-23 RTK, indicating an inhibitory role [90, 91]. Second, Cbl-N was found to associate with tyrosine-phosphorylated proteins [92, 93]. Third, a RING finger motif was identified in the central part of the protein that could functions as an E3 ubiquitin ligase [94-96]. These findings led to the hypothesis that Cbl

functions as a terminator of PTK signaling, mediating the ubiquitination and subsequent proteasomal degradation of tyrosine-phosphorylated proteins. Overexpression of Cbl induces polyubiquitination of RTKs such as EGFR, PDGFR, CSF-1R and c-MET, and was also shown to inhibit activated nonreceptor tyrosine kinases such as Src [97-102].

The ability of the N-terminal domain to bind tyrosine-phosphorylated proteins was explained by the X-ray crystal structure of Cbl-N, which revealed the presence of a divergent SH2 domain [103]. Although not recognizable by sequence analysis, this module has the unmistakable features of an SH2 domain, with a few variations (notably the absence of the βE and βF strands and of a prominent BG loop). The larger Cbl-N region, also termed the tyrosine kinase binding (TKB) domain, consists of a four-helix bundle, an EF hand calcium-binding motif, and the SH2 domain (Fig. 2B). This atypical SH2 domain serves as a reminder of the diversity of the SH2 domain family, which may have further unknown members that can only be recognized by secondary or tertiary structural analysis [104].

In the Cbl-N structure the four-helix bundle and EF hand contribute to the pTyr binding pocket of the SH2 domain, suggesting the three modules cooperatively recognize phosphorylated ligands, and indeed point mutation of any one of the three can abolish binding [103]. Screening of degenerate phophopeptide libraries indicated a consensus of D(N/D)x*po*Y for Cbl TKB binding, revealing the importance of N-terminal sequences and in particular the Y-2 residue (Fig. 1C) [105]. On the other hand, structural studies of Cbl-N bound to a ZAP-70 phosphopeptide indicated that the mode of interaction was similar to that of other SH2 domains, with the pTyr pocket and hydrophobic groove playing key roles. The groove is somewhat shallow, but specifically interacts with the Y+3 (Glu) Y+4 (Pro) residues of the ligand. Peptide residues N-terminal to pTyr were poorly ordered in the structure. Therefore, there is a discrepancy between

biochemical and structural data regarding importance of N-terminal and C-terminal residues for ligand specificity.

This discrepancy can be rationalized by carefully considering the experimental approaches. While randomized phosphopeptide library screening is a powerful tool to determine ligand preferences, results can be skewed by the use of biased libraries in which particular residues are not randomized. For instance, preference in the Y+4 position was not determined in the case of Cbl-N. Furthermore, this method can only provide the relative preference at each position, and cannot score the effect of combinations of residues at different positions. To address this issue, the direct sequencing of preferred peptides by mass spectrometry (MS) has been proposed [106-108]. The importance of sequence combinations is supported by the recent identification of an unrelated consensus binding site for the Cbl TKB in the c-Met family of RTKs. A D*po*YR (Y1003) motif, which clearly does not satisfy the previous Cbl TKB consensus, can bind to the domain and is important for Cbl-mediated downregulation of the receptor [109]. In this case molecular modeling experiments suggest an intramolecular salt bridge between the Asp and Arg residues of the motif may stabilize it in a conformation favorable for binding the pTyr pocket of the Cbl TKB domain. Nevertheless, both biochemical and structural data are consistent with other known physiological ligands for Cbl-N; for instance, SrcY416 (**DN**E*po*YTAAQQ) and EGFR Y1045 (LQR*po*YSSD**P**T) match the N-terminal and C-terminal consensus respectively (Fig. 1C) [110].

Another structural analysis, in this case of the Cbl TKB domain in conjunction with the RING finger and linker region, provided further clues to Cbl function [111]. Surprisingly, the RING finger was found to anchor the TKB domain, and the linker region to stabilize the association, suggesting that deletion of the linker region may cause dramatic conformational

instability of the TKB and RING finger [111]. The functional importance of this region is illustrated by the fact that 70Z-Cbl, which contains a deletion of only 17 amino acids in the linker region and RING motif (Fig. 2B), acquires oncogenicity [112]. Two tyrosine residues (Y368 and Y371) located in the middle of the linker region appear to be crucial. These tyrosines undergo phosphorylation under physiological conditions by Src and EGFR, and mutation of Y371 to Phe results in impaired ubiquitination of both PTKs [95, 113]. Presumably phosphorylation of Y368 and Y371 causes a conformational rearrangement of the TKB and RING domains that promotes the polyubiquitination of substrates such as Src and EGFR [114, 115].

Src family kinases associate with Cbl via their SH3 domains, which bind proline-rich motifs of Cbl. Accumulating data suggest Cbl can inhibit Src and its relatives by two distinct mechanisms, one via the RING finger and another via the TKB domain [114, 116]. Polyubiquitination of Src requires the kinase activity of Src and phosphorylation of Y371 in the linker region of Cbl, but not the Cbl TKB domain [113]. On the other hand, Cbl can also suppress Src kinase activity by the direct interaction of its TKB domain with phosphorylated Y416, within the activation loop of the Src catalytic domain [113]. Cbl is also known to associate with and down-regulate the ZAP-70 and Syk nonreceptor tyrosine kinases via its TKB domain, though the precise mechanism is not clear [100, 101, 117].

The mechanism of cell transformation by oncogenic Cbl is still not fully understood. Given that removal of the RING finger or linker region impairs the inhibitory role of Cbl (by abrogating its ubiquitin ligase activity), it was proposed that v-Cbl may act as a dominant negative, protecting phosphorylated substrates from endogenous Cbl and thereby leading to sustained PTK activation and cell transformation [118, 119]. In this model, oncogenicity requires

both an intact TKB domain and loss of the RING finger/ubiquitin ligase activity. Indeed, mutation of the TKB domain abolishes the transforming activity of v-Cbl [118]. However, the Y371F linker domain mutant of Cbl, which has lost the ability to ubiquitinate EGFR while retaining a functional TKB domain, fails to transform. Several other linker domain mutants similarly lose ubiquitin ligase activity yet are non-oncogenic, while others, such as Y371A and ΔY368, are as transforming as 70Z-Cbl [120]. These data indicate that dysfunction of the RING finger in conjunction with a functional TKB domain is not entirely sufficient for oncogenicity, suggesting additional regulation involving the linker region, e.g. interaction with other SH2-containing proteins or other regulators. On the other hand, it is now evident that oncogenic activation of RTKs, as in Tpr-Met and v-Fms, correlates with their inability to recruit Cbl as a result of mutation or deletion of TKB binding sites (reviewed in [121]).

2.3 Inducible suppressors: SOCS proteins

Although cytokine receptors do not possess intrinsic tyrosine kinase activity, their downstream signaling exploits SH2 domain-phosphotyrosine interactions. The cytoplasmic portions of these receptors constitutively associate with non-receptor PTKs of the JAK family. Upon ligand stimulation, homo- or heterodimerizion of receptors induces transactivation of JAKs, which in turn generates multiple tyrosine-phosphorylated sites on the receptor, providing binding sites for SH2 proteins such as Signaling Transducers and Activators of Transcription (STATs). STATs undergo tyrosine phosphorylation by JAKs, homodimerize via SH2-pTyr interactions, and subsequently translocate to the nucleus, where they induce transcription of cytokine-responsive genes [122]. In this system dissociation of STATs from the activated receptor is required. This is controlled by the affinity of STAT SH2 domain-ligand interactions, such that the affinity of the reciprocal STAT dimer is higher than that of the receptor for the

STAT SH2 [123]. The importance of JAKs and STATs for cytokine signaling is indicated by the fact that knockout mice for these genes fail to respond to specific cytokines [124].

In addition to activation, suppression and termination of cytokine signaling are also essential for appropriate immune and inflammatory responses [125, 126]. Negative regulation of cytokine signaling involves receptor internalization and degradation, tyrosine phosphatases, inositol phosphatases, Cbl (see Section 2.2, above), and Protein Inhibitors of Activated STATs (PIAS) [127-131]. Recently, the SOCS family of SH2 proteins has been added to this list [132-134]. Cytokine-inducible SH2 containing protein (CIS) was the first member of the family identified, as an immediate-early gene product induced by IL-2, IL-3 and erythropoietin (EPO) [135]. Upon EPO receptor stimulation, activated STAT5 binds the CIS promoter and induces its expression. In a classical negative feedback loop, CIS then binds to phosphorylated Y401 of the EPO receptor, the binding site for STAT5, thereby suppressing STAT5-mediated signaling [136]. Subsequently three groups identified proteins similar to CIS, termed Suppressor Of Cytokine Signaling-1 (SOCS-1), JAK-Binding protein (JAB), and STAT-induced STAT Inhibitor-1 (SSI-1) [137-139]. We now know that there are more than 40 molecules in a family defined by the presence of a "SOCS box," which is part of an E3 ubiqitin ligase complex that can target proteins for degradation via the ubiquitin proteasome pathway [140]. Eight of these, referred to here as SOCS proteins, possess an SH2 domain N-terminal to the SOCS box (Fig. 2C) [141-143].

Although SOCS proteins are similar in structure, varying mainly in the length of the region N-terminal to the SH2 domain, their biological functions are diverse [134, 144]. For example, SOCS-1 knockout mice exhibit constitutive activation of STAT-1 and overexpression of interferon-γ-induced genes, while SOCS-3 knockout mice exhibit severe erythrocytosis in

various organs [139, 145, 146]. While the SOCS box is interchangeable among different family members [140], two factors may account for the functional diversity SOCS proteins. One is the kinetics of their regulation in response to cytokines. For instance, IL-6 stimulation of mouse M1 cells induces expression of SOCS-1, but not SOCS-2 and SOCS-3. On the other hand IL-6 stimulation of mouse liver induces SOCS-1, -2, and -3 and CIS. SOCS-1 and SOCS-3 protein declines to basal levels within 4-8 hours, whereas CIS and SOCS-2 remain elevated over 24 hours [137].

A second factor is the contribution of sequences adjoining the SH2 domain. For instance, inhibition of JAK2 by SOCS-1 depends on two short sequences adjacent to the SOCS-1 SH2 [147-149]. One 12-amino acid stretch N-terminal to SH2 domain, termed the Extended SH2 Subdomain (ESS), stabilizes the association of SOCS-1 with the kinase activation loop of JAK2. An additional 12-amino-acid N-terminal segment, termed the Kinase Inhibitory Region (KIR), is required to inhibit of JAK2 kinase activity (Fig. 2C). Interestingly, KIR may act as a pseudosubstrate for JAK2 [147, 149]. A similar mode of inhibition has been observed in the Grb7, Grb10, Grb14 adaptor family proteins, which possess a PH domain and an SH2 domain. In the vicinity of their SH2 domains, a region termed BPS (between PH and SH2) or PIR (phosphotyrosine interacting region) binds the kinase domain of insulin receptor and suppresses its catalytic activity in cooperation with the SH2 domain [150-152]. Analogous to the autoinhibition of Src family PTKs by their own SH2 and SH3 domains, the SOCS and Grb7 families thus represent different modes of PTK inhibition in which the SH2 domain plays a central role [153].

In the case of the SOCS family, further diversity in function is provided by SH2 domain specificity. While SOCS-3 has a similar domain composition to SOCS-1, the SOCS-1 SH2 binds

JAKs rather than receptors, while the SOCS-3 SH2 binds receptors rather than JAKs [144]. The inhibitory effect of SOCS-1 on leukemia inhibitory factor (LIF) signaling is attenuated by replacement of its SH2 domain with that of SOCS-3 [149]. Interestingly, SOCS-1 loses its inhibitory activity by mutation of the invariant Arg in the conserved FLVRES motif in the βB strand of the SH2 domain, while SOCS-3 is tolerant to this mutation; for most SH2 domains, mutation of this residue abolishes binding to tyrosine-phosphorylated peptides. Taken together, these findings imply important functional difference between SH2 domains specific to particular cytokine pathways.

Regulation of IL-6/gp130 receptor signaling by SOCS-3 illustrates the complicated interplay between SH2 proteins and their binding sites. IL-6 stimulation leads to tyrosine phosphorylation of sites on the cytoplasmic tail of gp130, including Y767, Y814, Y905, and Y915. All of these sites match the STAT3 SH2 binding consensus (YxxQ), suggesting the involvement of STAT3 in downstream signaling [154]. On the other hand, Y759 (Y757 in mice) has been shown to play an important role in inhibition of STAT3 signaling and activation of Ras-MAPK signaling, as point mutation of this residue enhanced the IL-6 response and reduced MAPK activation [155-157]. Knock-in mice in which this site is mutated ($gp130^{Y759F}$) exhibit T cell hyper-responsiveness and rheumatoid arthritis (RA)-like joint disease within the first year of age [158, 159]. The SHP-2 tyrosine phosphatase is known to associate with phosphorylated Y759 in response to IL-6, suggesting SHP-2 is the negative regulator [155-157]. Recent data, however, demonstrate that SOCS-3 also inhibits IL-6 signaling via Y759, arguing that inhibition might in fact be due to SOCS-3 rather than SHP-2 [160]. As expected, the SH2 domains of SHP-2 and SOCS-3 favor similar motifs; Y759 (TVQ*po*YSTVVH) matches the consensus motifs for both (Fig, 1C) [157, 161]. Indeed, in vitro data revealed both proteins can bind the Y759 peptide

with similar affinity (K_d=140 nM for SOCS-3 vs. 170 nM for SHP-2), suggesting in vivo competition is likely [161].

One study demonstrated that the inhibitory effect of SOCS-3 was seen in cells lacking intact SHP-2, while conversely the forced localization of SHP-2 to the membrane in Y759F-expressing cells, in which association of SOCS-3 is prevented, also had an inhibitory effect [162]. Others also reported independent roles for SHP-2 and SOCS-3 in LIF-gp130 signaling using antisense oligonucleotides [163]. While these data indicate that SHP-2 and SOCS-3 independently regulate gp130 signaling via Y759, the physical and functional interaction of SOCS-3 with SHP-2 has also has been reported [162, 164]. In vitro binding experiments suggest that the SOCS-3 SH2 domain binds Y542 of SHP-2 [162], but since both molecules contain SH2 domains and potential tyrosine phosphorylation sites, and SHP-2 is known to engage in intramolecular SH2-pTyr interactions [165, 166], it is difficult to define the precise binding mode for the complex. To complicate matters even further, Ras-GAP, an SH2-containing negative regulator of the Ras pathway, was shown to bind Y221 in the C-terminus of SOCS-3 in response to IL-2, resulting in activation of Ras-MAPK signaling [167].

Taken together, signaling downstream of the gp130 receptor is shaped by the tyrosine phosphorylation of binding sites for the SH2 domains of STAT3, SHP-2 and SOCS-3. The physiological role of the dual regulation by Y759 is not clear at this moment. Recently Fischer et al. showed that pre-stimulation of cells with IL-6 renders STAT3 activation less sensitive to further stimulation with IL-6 [168]. Interestingly, this desensitization was not observed in SOCS-3 deficient cells, but was unaffected in cells expressing mutant SHP-2, indicating different qualitative roles for SOCS-3 and SHP-2. Whereas the IL-6-induced anti-inflammatory responses of SOCS-3 knockout macrophages and $gp130^{Y759F}$ mutant macrophages were similar [169], mice

lacking SOCS-3 in myeloid cells do not exhibit the severe RA-like inflammatory disease seen in gp130^{Y759F} transgenics [159, 169, 170]. Thus SHP-2 and SOCS-3 appear to exert distinct regulatory roles on the STAT pathway in vivo depending on the particular cell type.

3. SH2 domain inhibitors

Because of the important role of SH2 domains in signal transduction pathways, SH2-mediated protein-protein interactions are attractive therapeutic targets [171]. The fact that short pTyr-containing peptides (usually 5-6 amino acids) are sufficient to compete with larger protein ligands for SH2 domain binding has prompted researchers both in academia and industry to develop inhibitors targeting the SH2 domains of clinically relevant proteins, including Grb2, Src, Lck, Zap70, PI3K, and STAT3 [11, 172, 173]. A major difficulty in designing such inhibitors is the concentrated negative charge of SH2 ligands (from phosphate and acidic side chains), which is unfavorable for penetrating the cell membrane; in general, substitution of these negatively charged groups causes a dramatic loss of affinity [174, 175]. Elimination or modification of the bridging oxygen of phosphotyrosine (to confer phosphatase resistance) also adversely affects binding. Moreover, because the pTyr pocket of the SH2 domain is so well conserved, any inhibitor with high affinity to this site cannot be selective for a particular SH2 domain, unless it also targets specificity-determining regions. For all these reasons, developing high-affinity SH2 inhibitors that are specific, stable, and cell-permeable is a formidable challenge. Despite these difficulties, however, great progress is being made, and in this section we review efforts aimed at developing inhibitors of the Grb2 and Src SH2 domains. Comprehensive reviews of this field are also available [172, 176-179].

3.1 Inhibition of Grb2 SH2 domain

Since Grb2 is known to play a crucial role in signaling from RTKs to the Ras-MAPK cascade [180], Grb2 SH2 inhibitors would be expected to suppress cancer cell growth mediated by activated receptors such as HER2/Neu, EGFR, and c-Met. Grb2 is an adaptor protein possessing a single SH2 domain flanked by two SH3 domains. Upon RTK activation, the Grb2 SH2 domain preferably binds *po*YxNx motifs [7], such as Y317 of Shc and Y1139 of Her2/Neu, thus recruiting the Ras activator SOS to the membrane via the Grb2 SH3 domains [181, 182]. Crystal structures of the Grb2 SH2 bound to its cognate peptide ligand have revealed that the ligand adopts a type I β-turn conformation [26], whereas more typical SH2 ligands adopt an extended mainchain conformation [24, 25, 183] (see Section 1). These two unique characteristics--the strong requirement for Asn at the *po*Y+2 position of the ligand and the β-turn backbone conformation--suggested it would be possible to design highly specific inhibitors for the Grb2 SH2 domain.

3.1.1 Stepwise substitution of po*YxN motif*

A group from Novartis performed a systematic stepwise substitution at each residue of the parent tripeptide Ac-*po*YIN-NH_2, the shortest peptide retaining micromolar affinity to the Grb2 SH2 domain (blocked at the N and C termini with acetyl and amide groups, respectively) [184]. In this process, each position of the peptide is treated as a "module" which can be manipulated to mimic binding interactions observed in the crystal structure [26, 172, 176] (Fig. 3A). For instance, the *po*Y+1 residue must adopt a right-handed 3_{10} helical backbone conformation to stabilize the type I β-turn and maintain multiple van der Waals contacts with the SH2 surface. To meet these criteria, various α,α-substituted amino acids known to stabilize the 3_{10} helical conformation were docked by computational modeling, and the most favorable were

incorporated into the peptide and tested for binding affinity [185, 186]. Modeling predictions were consistent with results from competitive binding assays; for example aminocyclohexanecarboxylic acid (**Ac_6c**), predicted by modeling to have optimal van der Waals contacts, had the highest affinity among related analogs (Fig. 3B).

The same strategy was employed for other parts of the Ac-*po*YIN-NH_2 peptide to judiciously substitute peptide backbone and/or side chains. As Grb2 SH2 strongly favors Asn at the Y+2 position, any substitution at this position must fulfill strict local requirements, including a rigid backbone to stabilize the β-turn, preserving specific interactions with the LysβD6 and LeuβE4, and steric accommodation of TrpEF1 of the Grb2 SH2. cis-2-amino-cyclohex-3-ene carboxylic acid (**Achec**) was selected by substructure chemical database screening, and as predicted the 1S,2R diastereomer could substitute for Asn with little loss of affinity (Fig. 3B) [187]. The strict spatial requirements for this position are illustrated by the dramatic loss of binding potency seen with its diastereomer **(1R,2S)-Achec**. Because the crystal structure indicated that the C-terminal part of Ac-*po*YIN-NH_2 makes van der Waals contacts with a hydrophobic patch on the surface of the SH2 domain, a lipophilic attachment was designed. Appending a C-terminal 3-naphthalen-1-yl-propyl functionality improved the binding affinity by 25-fold [188]. Modifications N-terminal to pTyr, e.g. attaching 3-aminibenzyloxycarbonyl group (**3-amino-Z**) directly to the N-terminus, also led to dramatic improvements in binding potency (Fig. 3B).

All these substitutions were then combined to generate novel β-turn mimicking platforms, which were tested for in vitro binding affinity and for potency in cell-based assays [172, 176]. One such compound, **CGP78850** (Fig. 3C), is 200-fold more potent than the parent Ac-*po*YIN-NH_2 peptide, and could block EGFR-Grb2 and Shc-Grb2 interactions in MDA-MB-

468 human mammary carcinoma cells. More importantly, it also inhibited anchorage-independent growth of these cells, one of defining properties of tumor cells [189]. CGP78850 was further modified into a prodrug in which the pTyr phosphate was esterified to improve cell permeability. The prodrug version reverted morphological transformation of Her2/Neu-expressing NIH-3T3 cells and inhibited hepatocyte growth factor (HGF)-induced tumor cell motility [189, 190].

3.1.2 pTyr mimetics and macrocyclization

Burke and coworkers used the β-turn-mimicking platform of Novartis (above) as the starting point for two additional modifications, namely incorporation of pTyr mimetics and macrocyclization of the linear backbone. Because the cytosol has very high levels of constitutive tyrosine phosphatase activity, the stability of the phosphate linkage is a major concern in vivo. Early work indicated that substitution of pTyr with phosphonomethyl phenylalanine (**Pmp**), in which the pTyr bridging oxygen is substituted by a methylene group, led to a five-fold loss of binding potency in the case of ligands for the PI3K SH2 domain [191] . This was thought to be due to insufficient ionization of Pmp (because of its higher pK_a relative to pTyr at physiological pH), or the loss of hydrogen bonds between the SH2 and the pTyr bridging oxygen [192, 193]. When Pmp was incorporated into the β-turn platform, however, unexpectedly high affinity was reproducibly observed, comparable to that seen with pTyr (Fig. 3B) [176, 194]. This group also tested pTyr mimetic groups lacking phosphate, such as mono- or di-carboxy phenylalanine derivatives, in the β-turn platform [195, 196]. The monocarboxy series, e.g. carboxymethylphenylalanine (**cmF**, Fig. 3B), while useful in decreasing the net negative charge relative to pTyr, decreased affinity by almost a hundred-fold, underscoring the importance of two negative charges for molecular recognition by SH2 domains. Consistent with this, compounds

containing dicarboxy pTyr mimetics such as *p*-malonylphenylalanine (**Pmf**, Fig. 3B) displayed comparable affinity to those with Pmp or pTyr.

In order to stabilize the β-turn conformation, the Burke group also attempted to constrain the linear peptide mimetic by macrocyclization [197-199]. Surprisingly, although the cyclic analogue exhibited approximately a hundred-fold enhancement in binding potency in vitro relative to its linear counterpart, it was not effective in whole cell assays [197]. However, when the pTyr α position was modified by a carboxymethyl (CH_3CO_2H) functionality (Fig. 3C, **compound 1**), this compound not only blocked the association of Grb2 with Her2/Neu in whole cell assays, but also displayed anti-mitogenic effects in MDA-MB-453 cells [198, 200].

3.1.3 Non-phosphorylated ligand G1

Roller and coworkers employed a completely different strategy to identify novel unphosphorylated high-affinity ligands for the Grb2 SH2 domain [201]. They screened a combinatorial peptide phage-display library, where nine variable amino acids were flanked by cysteine residues (to promote disulfide-mediated cyclization). From this screen they isolated the cyclic peptide **CELYENVGMYC** (**G1**), which potently blocked binding of the Grb2 SH2 domain to a phosphorylated Shc Y317-derived peptide (IC_{50}=10-25μM) [201] (Fig. 3D, **G1TE**: thioether-bridged analogue of G1). G1 is not tyrosine-phosphorylated but does contain a YxN motif. Mutational studies indicated the particular importance of this motif, as well as Glu at the Y-2 position (Fig. 3D) [201, 202]. Structural analysis suggested that this cyclic peptide, for which ring closure is critical for binding, adopts a β-turn-like conformation similar to that found in natural (pTyr-containing) ligands, but utilizes a larger binding surface [202, 203].

Relative to physiological Grb2 binding sites (e.g. Y317 in Shc and Y1139 in ErbB2), where the residue at the -2 position is Pro, Glu at this position of G1 is unique. A Glu to Ala

substitution at this position of G1 caused a dramatic loss of affinity [201, 202, 204], while on the other hand substitution with unnatural amino acids containing acidic dicarboxylic side chains, e.g. γ-carboxyglutamic acid (Gla), significantly improved binding [204, 205]. Tyrosine phosphorylation of G1 increases binding affinity by more than 100-fold. Interestingly, in this context an acidic residues at the -2 position is no longer beneficial, and the Glu to Ala substitution actually enhances binding, which suggests complementary roles for phosphotyrosine or an acidic residue at the -2 position. Although no structural comparison is yet available, a plausible interpretation is that the carboxyl group of Glu can engage in some of the same interactions as the pTyr phosphate. This suggests some degree of plasticity in the SH2 domain, allowing multiple modes of binding with different ligands.

Upon optimizing each position of G1, a high-affinity Grb2 SH2 inhibitor peptide was produced (Fig. 3D **compound 2**; IC_{50}=26 nM) [205]. Importantly, compound 2 effectively inhibited the association of Grb2 with ErbB2 in whole cell assays at low micromolar concentrations, demonstrating the potential usefulness of non-phosphorylated SH2 inhibitors in vivo [205]. Alternatively, a G1 analogue containing the pTyr mimetic Pmp (thereby acquiring additional negative charge), which was further modified to increase cell permeability by attaching a hydrophobic carrier peptide, also exhibited anti-mitogenic effects in MDA-MB-453 cells (40% growth inhibition at 5μM) [206]. In addition, a compound incorporating non-phosphorylated 3-aminotyrosine was recently reported to have low nanomolar affinity for the Grb2 SH2 [207]. Thus it is apparent that there are multiple solutions to the tradeoff between the need for negatively charged pTyr mimetics for optimal affinity, and the need to minimize charge to facilitate cell entry.

3.1.4 Impact of N-terminal modifications

Although the two different Grb2 inhibitor platforms (the β-turn mimicking tripeptide and cyclic peptide G1) were developed independently, some similarities are apparent (Fig. 3C and D). For instance, incorporating aminocyclohexanecarboxylic acid (**Ach/Ac6c**) into the Y+1 position (which helps stabilize the β-turn conformation) and structural constraint via macrocyclization are beneficial in both cases. It also is noteworthy that modifications N-terminal to pTyr significantly improve binding affinity for both platforms. An early hint was provided by the observation that a phosphopeptide (E*po*YIN-NH_2) bearing an N-terminal fluorophore (anthranic group: **Abz**, Fig. 3B), which was used as a reference for ELISA-type assays, unexpectedly displayed a dramatic enhancement of affinity. This led to design of a 3-aminobenzyloxycarbonyl (**3-amino-Z**) N-terminal group [184]. Structural studies revealed stacking interactions between the electron-rich anthranyl moiety in the ligand and the electron-deficient guanidinium group of Arg αA2 of the SH2 [176, 208]. N-oxalyl modification (COCOOH-) also modestly improved affinity in the β-turn platform (Fig. 3B). Molecular modeling dynamics simulation studies suggested additional electrostatic interactions between the acidic oxalyl functionality and the positively charged sidechain of Arg αA2 [194, 195, 209].

N-terminal modifications also illustrate the complexities and subtleties inherent in "rational" inhibitor design. For instance, it is apparent that there are interesting compatibility issues between specific N-terminal modifications and pTyr mimics. For example the Nα-3-amino-Z modification improves binding for pTyr-containing ligands, but not for Pmp-containing ligands [209], while the Nα-oxalyl functionality is compatible with both pTyr and Pmp [194, 209]. It is also remarkable that N-terminal modification by oxalyl or carboxymethyl (CH_2COOH-) functionalities brings an additional negative charge to the corresponding inhibitors; despite this, such compounds are often active in cell-based assays [197, 198]. The

affinity improvement observed in in vitro assays may not entirely account for this effect, since the unmodified counterparts, which are inactive in cell based-assays, already bind with high affinity.

Importantly, N-terminal modification generates additional binding interactions not observed in the crystal structure of the Grb2 SH2 bound to peptides derived from natural ligands. In the case of the non-phosphorylated cyclic G1 peptide, structural modeling analyses indicated acidic sidechains at the Y-2 position make multiple interactions with arginines and serines at the pTyr pocket [204]. Do any natural ligands take advantage of similar N-terminal interactions? Rojas et al. showed that a peptide encompassing the Shc-Y317 phosphorylation site (FDDPSYVNVQNL), which has acidic residues at the Y-3 and Y-4 positions, could inhibit Grb2-Shc interaction when delivered into cells without prior phosphorylation [210]. Thus under physiological conditions, it is likely that binding of Grb2 to sites such as Shc-Y317 would be less dependent on tyrosine phosphorylation than more typical SH2 interactions.

3.2 Src inhibition and bone resorption

Src is the prototype nonreceptor PTK, and its activation in human tumors such as breast cancer has been reported [211-213]. Src-deficient mice exhibit osteopetrosis, or hypertrophy of bones, indicating impaired osteoclast function [214]. Therefore, antagonizing Src function in vivo may inhibit tumor growth and be useful for treatment of osteoporosis and prevention of osteolytic changes related to tumor metastasis. However, because the Src protein is tightly regulated by multiple domains including SH2, SH3 and kinase domains, the consequences of inhibition of the Src SH2 domain are not entirely straightforward. Non-receptor tyrosine kinases generally require SH2 domains for efficient substrate phosphorylation [20], so SH2 inhibition is likely to suppress phosphorylation and downstream signaling. In addition it may suppress kinase-

independent adaptor functions mediated by SH2 and SH3 domains of Src [215]. On the other hand, Src is normally held in an inactive conformation via an intramolecular interaction between its SH2 domain and a tyrosine-phosphorylated C-terminal residue, Y527. Abrogation of this interaction leads to activation of the kinase, and Y527 mutants of Src, e.g. Y527F, are known to be transforming. Hence Src SH2 inhibitors are potentially valuable, but any clinical applications must take into account the potentially disastrous effects of inappropriate activation of Src.

Nevertheless, Src SH2 inhibitors have great potential to treat osteoporosis. One reason for optimism is that knock-in mice bearing a kinase-inactive K295M Src mutant exhibit partial recovery of the osteopetrosis phenotype, indicating that the Src SH2 and SH3 domains play a critical role in osteoclast function [215]. Until now, osteoporosis has generally been treated with two categories of drugs: bone resorption inhibitors such as biphosphonate, and stimulants of bone formation, such as parathyroid hormone and prostaglandin E2 [216]. In recent years several pharmaceutical companies including Glaxo, ARIAD and Aventis have been working to develop Src SH2 inhibitors, with the rationale that Src is a validated therapeutic target for bone resorption [178, 217, 218].

3.2.1 Importance of the scaffold

The Src SH2 binds to high-affinity ligands via a "two-pronged plug and socket," where the sidechains of pTyr and the Y+3 residue (generally Ile) are buried in distinct pockets [24, 25, 219]. There is no doubt that these two regions, with multiple specific contacts, are crucial for the SH2-ligand association. However the linker region between pTyr and Y+3 (Y+1 and Y+2 residues of a peptide ligand) is also important for the design of Src SH2 inhibitors. This region makes contacts with the solvent-accessible hydrophobic surface of the SH2 domain and acts as a scaffold that appropriately orients the pTyr and Y+3 sidechains into their respective pockets

[177, 220] (Fig. 4A). In the crystal structure of the Src SH2 domain bound to high affinity peptide, the mainchain of the Y+1 residue makes direct contact with HisβD4 of the domain, while the sidechains of Y+1 and Y+2 are relatively free [24]. Nevertheless a series of analogs containing dipeptides or hydrophobic linkers between pTyr and the Y+3 Ile displayed more than 100-fold lower affinity than the wild-type peptide [175]. Single amino acid substitutions at Y+1 or Y+2 were tolerated relatively well, but D-amino acid substitution for Y+1 caused a major loss of affinity, indicating the importance of the backbone conformation in this position [175]. The hydrophobic surface here is mainly formed by TyrβD5, which is known as a specificity-determining residue [6, 33]. Mutational studies have shown that substitution of TyrβD5 to Phe results in a 30-fold loss of affinity [221]. Surprisingly, this effect is greater than for a mutation of the conserved ArgαA2, known to make important electrostatic interactions in the pTyr pocket, which results in only a six-fold affinity loss [31]. TyrβD5 makes van der Waals contacts with the sidechain of Y+1, forming a platform for the extended ligand peptide backbone [24, 221]. Therefore, selection of a scaffold that favorably interacts with this hydrophobic surface is a critical element in designing inhibitors.

The ideal scaffold would provide a rigid backbone, preserve existing hydrogen bonds with HisβD4, LysβD6 and IleβE4, and make stacking interaction with the aromatic ring of TyrβD5 [222]. Researchers at ARIAD have conducted structure-based design studies examining various types of scaffolds for Src SH2 inhibitors, including ureido-type peptide mimetics [223], di-substituted thiazoles [224], oxazoles [220] and benzamide [225]. Notably, X-ray crystallography revealed the benzamide template forms hydrogen bonds with LysβD6, displacing water molecules found in the previous X-ray structure. Subsequently, a seven-membered bicyclic benzamide scaffold platform, which fully complements the convex surface

created by TyrβD5, has been developed [222] (Fig. 4B). Using an alternative approach, researchers at Aventis selected a caprolactam scaffold and also confirmed displacement of structured water molecules by X-ray crystallography. This platform, incorporating pTyr and biphenyl functionalities, showed 15-fold higher affinity than the natural *po*YEEI peptide [226].

3.2.2 Cysteine-targeting pTyr mimetics

In order to improve selectivity for the Src SH2 domain versus those of other structurally similar Src family members, a series of cysteine-targeting pTyr mimetics have been designed [227-229]. The Src SH2 has a cysteine at βC3 which is conserved in Src proteins of different species, but not among other Src family proteins, where the βC3 residues are serine. Even among the entire complement of human SH2 domains CysβC3 is rare, found only in SAP, EAT2, and Src (C. Thompson, unpublished observation). Bradshaw et al. suggested this cysteine is particularly reactive since the highly basic environment of the pTyr pocket lowers the pK_a of the sulfhydryl [174]. Therefore, an appropriately positioned electrophile such as an aldehyde group may form a reversible covalent bond with this group [227]. Various CysβC3-targeting pTyr mimetics including 3'-formyl-pTyr, 3'-formyl-4'-Pmp and 3'-formyl-4'-carboxy-phenylalanine have been tested. The 3'-formyl-4'-Pmp template did not engage CysβC3 because of the disposition of the aldehyde group [230]. For the 3'-formyl-pTyr template, X-ray structures confirmed hemithioacetal formation but no inhibition was observed in cell-based assays. This failure was most likely due to some combination of loss of affinity through entropic effects at the pTyr pocket, phosphatase susceptibility, and poor cell permeability [227, 228, 230].

On the other hand, a 3'-formyl-4'-carboxy-phenylalanine-containing (Fig. 4B, **Fcp**) SH2 inhibitor, **AP22161** (Fig. 4C), showed dramatic improvement in binding potency. AP22161 was shown to form a hemithioacetal by nuclear magnetic resonance (NMR) analysis, dependent on

interaction of the formyl group with CysβC3. It binds the Src SH2 100-fold better than that of Src family member Yes, and 1700-fold better than the more distantly related ZAP-70 SH2s [229] (Fig. 4C). Moreover, AP22161 inhibited rabbit osteoclast-mediated resorption of dentine and showed partial morphological reversion of Src Y527F mutant-transformed rat fibroblasts [229]. The normal physiological role of this highly reactive cysteine is not clear. Mutation of CysβC3 causes an eight-fold improvement in affinity for consensus ligands, suggesting a negative role in the pTyr pocket [31]. In addition, CysβC3 may play some role in the weak phosphatase activity reported for the Src SH2 domain [231].

3.2.3 Targeting SH2 inhibitors to bone

Another approach to enhance specificity is to target inhibitors to therapeutically relevant cells or tissues. Therefore attaching functionalities that specifically target Src SH2 inhibitors to bone might promote the inhibition of bone resorption by osteoclasts while minimizing potential undesirable effects on other cells. To test this concept, two bone-directed bisphosphonate-incorporating pTyr mimetics, 4'-diphosphonomethyl-phenylalanine (termed as **dpmF** or DMP) and 3', 4'-diphosphonophenylalanine (**Dpp**) have been incorporated into benzamide templates to generate compounds such as **AP22408** [232-234] (Fig. 4B and C). Here dpmF and Dpp have dual functions, providing selectivity for bone and binding to the pTyr pocket. Although the multiple phosphates of dpmF and Dpp create additional negative charge relative to pTyr, inhibitors incorporating these groups effectively suppress in vitro bone absorption by rabbit osteoclasts at reasonable concentrations (IC_{50}<2μM), indicating that they can enter the cell efficiently [232-234]. While direct experimental evidence is not available, a plausible explanation involves accumulation of these inhibitors in bone matrix followed by uptake into osteoclasts by phagocytosis [177]. Interestingly, bisphosphonates themselves are known to

inhibit osteoclast function and bone metastasis [235, 236]. The osteoclast inhibitory abilities of Dpp- and dpmF-incorporating SH2 inhibitors, however, depend on their binding potency to the Src SH2 domain [233].

The two classes of Src SH2 inhibitors discussed here, cysteine-targeting and bone-targeting compounds, displayed almost equivalent in vitro binding potency (IC_{50}=0.1-0.4μM), while the IC_{50} values for the rabbit osteoclast resorption assay were very different, 43μM for the cysteine-targeting compound and 2μM for dpmF-incorporating compound [229, 232]. To date an inhibitor with both cysteine- and bone-targeting functionalities has not been reported. It is important to note that proof-of-concept studies have demonstrated that Src-specific SH2 inhibitors indeed suppress bone resorption by osteoclasts [229, 232-234]. Since Src kinase inhibitors display similar activity, the functional importance of both the SH2 and kinase domains of Src in osteoclasts has been convincingly demonstrated [237, 238].

3.3 Structure-based design and binding mode

It is obvious that computational modeling, taking advantage of available crystal structure data, plays a key role in the structure-based design of SH2 inhibitors. For example, inhibitors containing various scaffolds were docked onto the Src SH2 domain, using the pTyr residue as an anchor based on the known binding mode of *po*YEEI high-affinity peptides, leading to the choice of benzamide scaffolds [225]. However, subsequent X-ray crystal structures demonstrated significant differences in the details of the actual mode of binding compared to modeling predictions. These differences suggest difficulties in de novo prediction of binding mode by computational modeling, and emphasize the importance of confirmation by structural analysis for each SH2 inhibitor [223, 227]. Such structural studies sometimes reveal new and unexpected binding modes, which in turn provide valuable information for further inhibitor design. For

instance, crystallizing the Src SH2 domain in citrate buffer unexpectedly revealed a citrate ion stacking into the pTyr pocket [232]. Interestingly, additional binding interactions were seen, e.g. ionic bonds between citrate and LysβD6, that had not been noted in previous structural studies, leading to the design of new pTyr mimetics such as dpmF and Dpp [232-234].

Researchers from Aventis reported the superimposition of crystal structure data for eleven SH2 inhibitors, comprising two different scaffold series, bound to the Src SH2 domain [239]. This valuable study revealed three different ligand-binding modes to the SH2 domain, depending on the mode of interaction with the Y+3 hydrophobic pocket of the Src SH2 domain. Thus they suggested that the SH2 domain is able to adopt different binding modes, thereby increasing the flexibility of its interactions. This is most likely because the pTyr pocket can accept phosphate in different orientations, and also because ordered water molecules can bridge between scaffold or hydrophobic regions of the ligand and the rigid surface of the SH2 domain.

3.4 Bioavailability of SH2 inhibitors and other challenges

In light of the major constraints on the bioavailability of SH2 inhibitors (cell permeability, stability, and antigenicity), together with simplicity of synthesis, it is clear that smaller, less charged compounds with minimal peptidic character are ideal [240, 241]. In terms of size and peptidic character, Src SH2 inhibitors roughly the size of dipeptides have been developed with lipophilic scaffolds [178] (Fig. 4), while the Grb2 SH2 domain seems to require larger macrocyclic ligands to achieve high affinity (Fig. 3). The issue of negative charge is still challenging. Decreasing the charge of pTyr mimetics improves cell permeability, at the price of decreased affinity for the pTyr pocket. What is the minimum acceptable charge for binding to SH2 domains? Beaulieu et al. tested various substitutions for acidic side chains of a *po*YEEI-OH peptide for their effect on binding of the Lck SH2 domain, and demonstrated low micromolar

affinity for an inhibitor bearing a net charge of -2 at physiological pH [242]. Gratifyingly, the bone-targeting Src SH2 inhibitor (bearing the highly-charged bisphosphonate pTyr mimetic) is effective in cell-based assays; in this case the disadvantage of high net negative charge is offset by the advantage of bone-specific targeting [232-234]. In order to rapidly screen potential SH2 inhibitors and drive future development, high-throughput assay platforms that can simultaneously evaluate both binding potency and cell permeability will be critical. The ARIAD group has developed a promising mammalian cell assay based on the two-hybrid system and used it to screen a panel of Src SH2 inhibitors [234, 243].

A major rationale for developing both the Grb2 and Src SH2 inhibitors was their potential for treating human malignancies. Despite the spectacular recent success of Imatinib in treatment of chronic myelogenous leukemia and other malignancies [244], achieving a significant survival benefit with new anti-tumor agents remains a daunting challenge. Nygren et al. recently evaluated publicly available data for a total of 209 investigational anti-cancer drugs, of which only 12 were shown to have survival benefit in randomized controlled clinical trials [245]. To date Ras antagonists have demonstrated only limited efficacy, so the ultimate clinical success of Grb2 SH2 inhibitors, which target Ras signaling, is far from assured [245]. Furthermore, not surprisingly Grb2 inhibitors are ineffective in cells expressing mutationally activated Ras, because Ras is downstream of Grb2 in the signaling pathway [189]. It should be noted, however, that Grb2-SH2 inhibitors may block other pathway(s) normally mediated by the SH3 domains of Grb2, such as PI3K-Akt cell survival signaling. On the other hand, a Src SH2 inhibitor could partially revert cell transformation induced by Y527F Src, although a ten-fold higher concentration was needed relative to the Src kinase domain inhibitor PPI [234]. Moreover, a concern with Src SH2 inhibitors is their potential to inappropriately activate Src kinase by

competing away the autoinhibitory interaction between the Src SH2 and the regulatory pTyr. The Aventis group reported that several high-affinity Src SH2 inhibitors did not activate Y527-phosphorylated Src in ELISA-based assays and yeast whole cell assays; the reasons for this are unclear [246]. Despite these remaining unresolved issues, the concept of using SH2 inhibitors to specifically block phosphorylation-dependent protein-protein interactions remains an attractive one.

4. Concluding remarks

We have highlighted three SH2-containing proteins and two groups of SH2 inhibitors to illustrate recent progress in understanding both the common features of SH2 domains, and the differences that allow them to be adapted to so many cellular functions. These recent studies reveal a number of subtle twists on the common and accepted themes of SH2 function; for example previously unappreciated binding modes were discovered, underscoring the plasticity of SH2 domains which is likely to be relevant to interactions with physiological ligands. It is now apparent that the SH2 domain features an "adjustable fit" to accommodate various ligands, consistent with the notion that a single SH2 domain displays only modest specificity for various phosphopeptides in vitro assays [27]. It is of particular of interest to know in which situations SH2 domains utilize ambiguous molecular recognition, which may favor propagation of multiple signals, versus strict molecular recognition (achieved by tandem domain arrangement or high local concentrations of ligand) leading to a single specific output.

The increasing availability of genome-wide and proteome-wide data will greatly impact both our understanding and possible exploitation of SH2 domain-mediated signaling. Phosphoproteomic analyses will produce unprecedented details about the identity, abundance,

and kinetics of appearance of binding sites for different SH2 domains under various physiological conditions. Coupled with data on the binding preferences and abundance of all ~116 SH2 domains in the cell, we will be able to use computational modeling to predict which interactions will occur under different conditions, along with their subcellular localization and likely biological outputs. The first attempts have been made to profile the global patterns of SH2 domain binding sites in the cell [247, 248], and such approaches are likely to become more quantitative and high-throughput in the future. In addition to helping understand normal cell signaling, such SH2 profiles may serve to highlight and classify aberrant signaling, as seen in immune disorders and cancer. Given the likely future development of highly specific and cell-permeable SH2 domain inhibitors, this raises the prospect of using such inhibitors to therapeutically manipulate signaling pathways in the cell. The first tentative steps down this road have already been taken, and we can expect the pace of progress to redouble in the near future.

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Figure Legends

Figure 1. Common architecture of SH2 domains

A. Sequence alignment of various SH2 domains. Typical secondary structural elements domains are depicted above the sequences and labeled according to standard nomenclature; α helices are colored orange and β strands blue. Divergent SH2 sequences for Cbl and STAT1 are also shown. **B.** Ribbon diagram of prototypic SH2 domain. Secondary structural elements and loops are labeled. Figure was modified from crystal structure of Src SH2 domain [24]. **C.** Phosphopeptide preferences of SH2 domains. Sequence preferences of SH2 domains described above were determined by combinatorial phosphopeptide library screening ([6, 7, 56, 58, 105, 161, 249-252]), mutagenesis ([253-260]) or structural analysis ([103]). Positions are denoted as N-terminal (-3, -2, -1) or C-terminal (+1, +2, +3, +4, +5) relative to pTyr (0). X, any amino acid; Φ, hydrophobic amino acid. n.d., not determined (this position was not randomized or mutated in relevant reports).

Figure 2. Structure of SH2 domain-containing proteins

SH2 domain proteins discussed in this review are depicted diagramatically. **A.** SAP/SH2D1A consists of a single SH2 domain. EAT-2 also possesses a tyrosine phosphorylation site on its C terminus. **B.** Cbl family proteins consist of multiple functional domains: a Cbl-N or Tyrosine Kinase Binding (TKB) domain, which contains a four-helix bundle (4H), EF-hand (EF), and variant SH2 domain (SH2); linker region containing two putative tyrosine phosphorylation sites Y368 and Y371 (denoted as YY); Ring Finger domain (RF); Proline-Rich regions (PR); multiple tyrosine phosphorylation sites (denoted as YYY); and Leucine Zipper region (LZ). **C.** Eight

members of the Suppressors Of Cytokine Signaling (SOCS) family are characterized by a central SH2 domain and C-terminal SOCS box. SOCS-1 and SOCS-3 have additional motifs adjacent to the SH2 domain, the Extended SH2 Subdomain (ESS) and Kinase Inhibitory Region (KIR).

Figure 3. Structure-based design of Grb2 SH2 domain inhibitors

A. Schematic representation of binding mode of Grb2 SH2 domain with specific inhibitor. The inhibitor consists of five "modules" mimicking the parent tripeptide **Ac-*po*Y(I/V)N-NH_2.** The modules for the pTyr mimetic and Y+2 position anchor to their respective binding pockets on the surface of the domain, while an acidic or lipophilic modification N-terminal to pTyr creates additional binding interactions, Y+1 module promotes β-turn mainchain conformation, and the C-terminal module interacts with hydrophobic surface of the domain. Key residues in the domain for each position are depicted. **B.** Chemical structures of selected substituents for each position. *[1]Relative potency (in parentheses) is defined as IC_{50}-control/ IC_{50}-subject in equivalent inhibitor platform. *[2] IC_{50} values are compared in pTyr linear peptide platform [185]. Different values were reported in Pmp-based platform [209](see text, section 3.1.4). *[3] Direct comparisons of IC_{50} values in same platform are not available. Instead, 3-indolyl-1-yl-propyl, approximately 18 times more potent than 3-naphtalen-1-yl-propyl, has been reported [176, 188, 194, 196]. **C.** Chemical structures and IC_{50} values obtained from competitive binding assays. **Ac-*po*YIN-NH_2**, minimum tripeptide retaining micromolar affinity to Grb2 SH2 domain [184]; **CGP78850**, Pmp-incorporating β-turn-mimicking inhibitor platform [189]; **Compound 1**, β turn-stabilizing macrocyclic inhibitor with carboxylic functionality (-CH_2COOH) at Pmp α position [198]. **D.** Chemical structure and IC_{50} values of cyclic non-phosphorylated Grb2 SH2 ligand peptides **G1TE** and its modified form **Compound 2** [201, 205]. Abbreviations; **Ac**, acetyl group; **Abz**,

anthranic acid; **3-amino-Z**; 3-aminobenzyloxycarbonyl group; **pTyr**, phosphotyrosine; **Pmp**, phosphonomethyl phenylalanine; **cmF**, carboxymethylphenylalanine; **Pmf**, *p*-malonylphenylalanine; **Ac_nc** (n=5, 6, 7), a,a-disubstituted cyclic α-amino acids; **Achec**, cis-2-amino-cyclohex-3-ene carboxylic acid.

Figure 4. Structure-based design of Src SH2 domain inhibitors

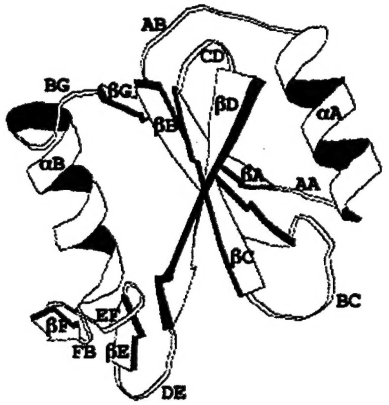
A. Schematic representation of binding mode of Src SH2 domain with specific inhibitor. The inhibitor consists of three components: pTyr mimetic, central scaffold and hydrophobic moiety. The central scaffold interacts with a hydrophobic surface formed by TyrβB5, and appropriately delivers pTyr mimetic and hydrophobic functionalities into their respective binding pockets. Key residues in the domain for each position are depicted. **B.** Chemical structures of selected substituents for each position. *[1]Relative potency is defined as in Fig. 2. Values for scaffolds are determined in combination with cyclohexyl (*[2]) or biphenyl (*[3]) modification for hydrophobic region respectively [222, 239, 246]. **C.** Chemical structures and IC_{50} values by competitive binding assay for Src SH2 inhibitors. **Ac-*po*YEEI-NH_2**, a tetrapeptide with micromolar affinity to Src SH2 domain [229]; Cysteine-targeting **AP22161**, 3'-aldehyde 4'-carboxyl phenylalanine Fcp-incorporating bicyclic benzamide scaffold template exhibiting selective Src SH2 binding potency [229]; Bone-targeting **AP22408**, Dpp-incorporating bicyclic benzamide scaffold template exhibiting selective inhibition of osteoclasts [233]. Other bone-targeting high-affinity Src SH2 inhibitors carrying dpmF (IC_{50}=0.35) have also been reported [232, 234]. Abbreviations; **pTyr**, phosphotyrosine; **Cdcp**, 3'-carboxyl 4'-dicarboxyl phenylalanine; **Fcp**, 3'-aldehyde 4'-carboxyl phenylalanine; **Dpp**, 3', 4'-diphosphonophenylalanine; **dpmF**, diphosphonomethyl-phenylalanine.

Fig. 1

A

```
Src      WYFGKI-TRRESERLLLNAENPRGTFLVRESETTK-GAYCLSVSDFDNAKGL--NVKHYKIRKLDSG----------------GFYITSR-------TQFN-SLQQLVAYYSKHADGLCH------RLTTVC
P85α_N   WYWGDI-SREEVNEKLRDTAD--GTFLVRDASTKMHGDYTLTLRK-----GG--NNKLIKIFHRD-G----------------KYGFSDP-------LTFS-SVVELINHYRNESLAQYNPKLDV-KLLYPV
Crk      WYWGRL-SRQEAVALLQGQRH--GVFLVRDSSTSP-GDYVLSVSENS-------RVSHYIINSSGPRPPVPPSPAQPPPGVSPSRLRIGD--------QEFD-SLPALLEFYKIHYLDTT-------TLIEPV
Grb2     WFFGKI-PRAKAEEMLSKQRHD-GAFLIRESESAP-GDFSLSVKF-----GN--DVQHFKVLRDGAG----------------KYFLWV--------VKFN-SLNELVDYHRSTSVSRNQQI----FLRDIE
Syk_C    WFHGKI-SREESEQIVLIGSKTNGKFLIRARDNN--GSYALCLLHE----G---KVLHYRIDKDKTG----------------KLSIPEG-------KKFD-TLWQLVEHYSYKADGLLR------VLTVPC
SHP-2_N  WFHPNI-TGVEAENLLLTRGVD-GSFLARPSKSNP-GDFTLSVRRN----G---AVTHIKIQNT--GD----------------YYDLYGG-------EKFA-TLAELVQYYMEHHGQLKEKNGDVIELKYPL
SOCS3    FYWSAV-TGGEANLLLSAEPA--GTFLIRDSSDQR-HFFTLSVKTQS-------GTKNLRIQCE--GG----------------SFSLQSDPRSTQPVPRFD-CVLKLVYHYMPPPGAPSFPSPPTEPSS
SAP      VYHGKI-SRETGEKLLLATGLD-GSYLLRDSESVP-GVYCLCVLYH----G---YIYTYRVSQTETG----------------SWSAETAPGVHK--RYFR-KIKNLISAFQKPDQGIVI------PLQYPV
Cbl      GYMAFLTYDEVKARLQKFIHKP-GSYIFRLSCTRL-GQWAIGYVTAD---G---NILQTIPHNKP-----------------------------------LFQALIDGFREGF----------YL-FPD
STAT1    CIMGFISKERERALLKDQQP---GTFLLRFSESSREGAITFTWVERSQNGGEPDFHAVEPYTK-------------------------------KELSAVTFPDIIRNYKVMAAENIPENPLKYLYPNIDKDHAFG
```

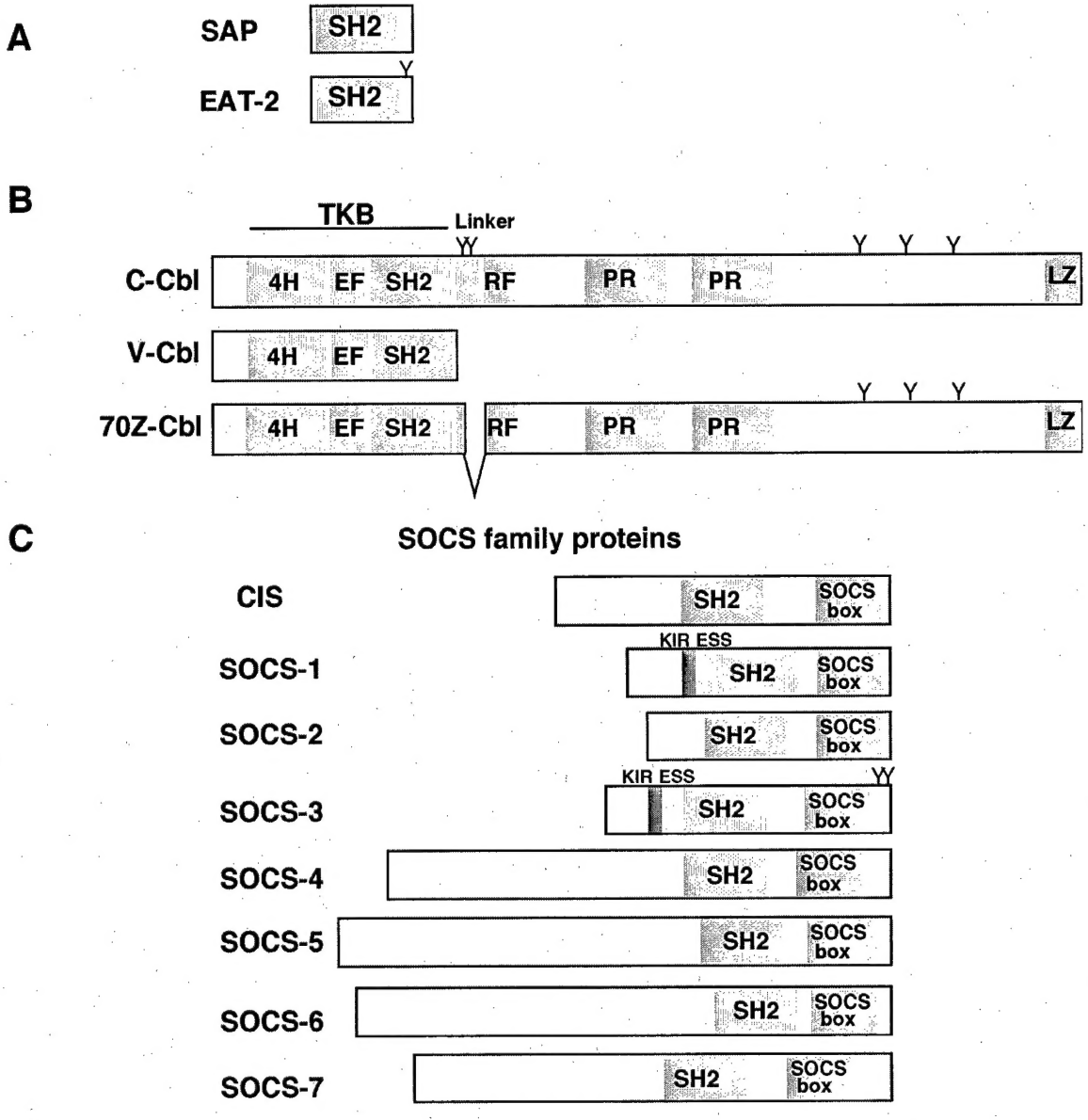
B



C

	SH2 domain	-3	-2	-1	0	1	2	3	4	5	ref.
1	Src	*n.d.*	*n.d.*	*n.d.*	pY	E	E	I	*n.d.*	*n.d.*	
2	p85α_N	X	X	X	pY	M/V	X	M	*n.d.*	*n.d.*	
3	Crk	*n.d.*	*n.d.*	*n.d.*	pY	X	X	P	*n.d.*	*n.d.*	
4	Grb2	*n.d.*	*n.d.*	X	pY	X	N	X	X	X	
5	Syk_C	*n.d.*	*n.d.*	X	pY	E	X	L	X	X	
6	SHP-2_N	X	V	X	pY	I/V	X	V/I/L	X	W/F	
7	SOCS-3	*n.d.*	*n.d.*	*n.d.*	pY	V/A/Y	Φ	V/I/L	Φ	H/Y/V	
8	SAP	X	T/S	I	(p)Y	X	X	V/I	X	*n.d.*	
9	Cbl	D	N/D	X	pY	X	X	X	P/Φ	*n.d.*	
10	STAT1/3	X	X	X	pY	X	X	Q	X	X	

Fig. 2


A
SAP
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EAT-2
SH2
Y
B
TKB
Linker
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EF
SH2
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PR
LZ
V-Cbl
4H
EF
SH2
70Z-Cbl
4H
EF
SH2
RF
PR
PR
LZ
C
SOCS family proteins
CIS
SH2
SOCS box
SOCS-1
KIR ESS
SH2
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SH2
SOCS box
SOCS-3
KIR ESS
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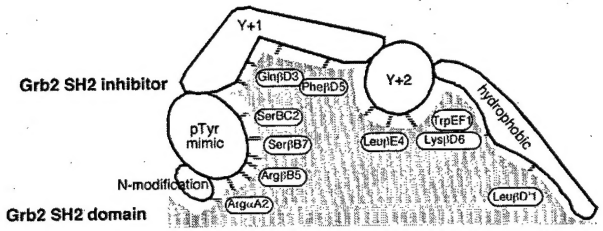
Fig. 3

A



B

N-modification	pY (mimic)	Y+1	Y+2	hydrophobic
Ac (=1)	**pTyr** (=1)	**Val** (=1)	**Asn** (=1)	**NH$_2$** (=1)
Abz (Ac X392*2)	**Pmp** (pTyr X1.23)	**Ac$_5$c** (Val X0.54)	**(1S,2R)-Achec** (Asn X0.65)	
3-amino-Z (Ac X132*2)	**cmF** (pTyr X0.02)	**Ac$_6$c** (Val X20.4)	**(1R,2S)-Achec** (Asn X0.0002>)	**3-naphtalen-1-yl-propyl** (NH$_2$ X26.9)
oxalyl (Ac X3.25)	**Pmf** (pTyr X0.37)	**Ac$_7$c** (Val X3.87)		**3-indolyl-1-yl-propyl** (NH$_2$ X~486*3)

(Values are relative potency: IC50-control/ IC50-subject*1)

C

Ac-pYIN-NH$_2$
IC$_{50}$=8.6 μM

CGP78850
IC$_{50}$=0.043μM

compound 1
IC$_{50}$=0.002μM

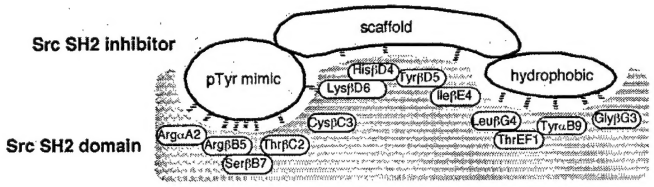
D

G1TE
IC$_{50}$=20μM

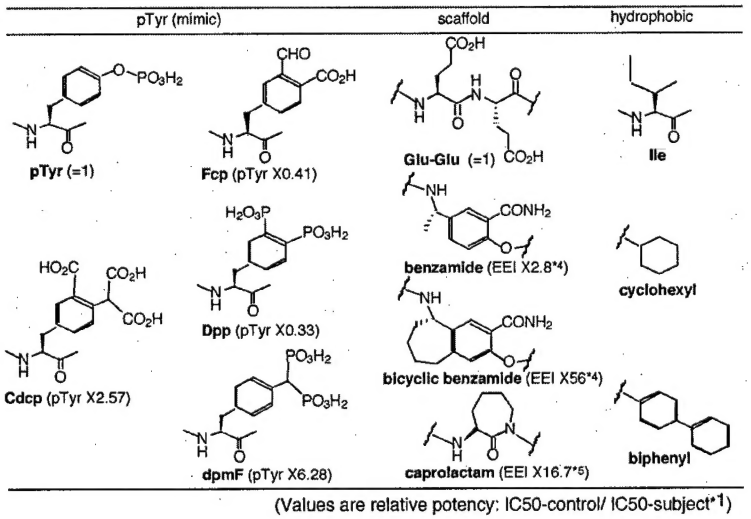
compound 2
IC$_{50}$=0.026μM

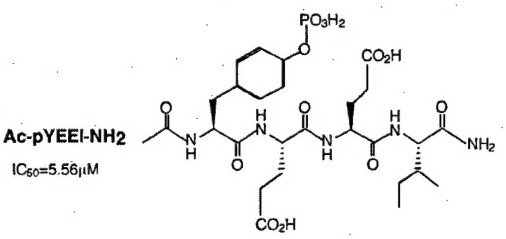
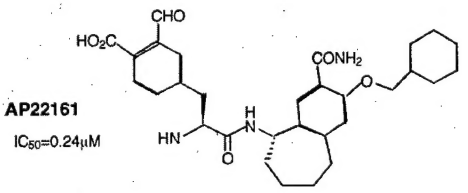
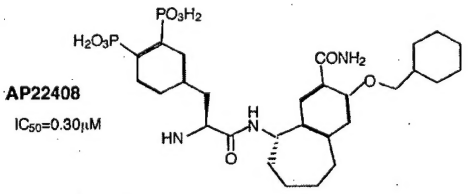
Fig. 4

A



B



(Values are relative potency: IC50-control/ IC50-subject*1)

C